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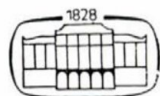
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INDEX TO VOLUME 35

NUMBERS 1—4

NUMBER 1

The Structure of O-Specific Polysaccharides and Serological Classification of <i>Pseudomonas aeruginosa</i> (A Review)	
Knirel, Yu. A., Vinogradov, E. V., Kocharova, N. A., Paramonov, N. A., Kochetkov, N. K., Dmitriev, B. A., Stanislavsky, E. S., Lányi, B.	3
Cytotoxic Activity of Lymphocyte Subpopulations Against Autologous Tumour Cells in Patients with Myeloid Leukaemias and Preleukaemic Disorders	
Szabó, B., Váczi, L., D. Tóth, F., Kiss, J., Kiss, A., Rák, K.	25
Production of Toxic Shock Syndrome Toxin-1 (TSST-1) Associated with <i>Staphylococcus aureus</i> from a Pyogenic Skin Infection	
Naidu, A. S., Rathna, K., Naidu, S., Rao, P. N., Rajyalakshmi, K.	35
Imprinting by Non-Signal Protein Molecule in <i>Tetrahymena</i>	
László, V., Csaba, G., Vargha, P.	41
Enhancement of Cilia Regeneration by Hormone Treatment of <i>Tetrahymena</i>	
Darvas, Zs., Árvai, G., Csaba, G., Vargha, P.	45
Bacterial Translocation in Dianhydrotolcitol-Treated Mice	
Anderlik, P., Szeri, I., Bános, Zs.	49
A Phage Typing System for <i>Salmonella infantis</i>	
G. László, V., Csák, K., Sz. Csórián, E.	55
A Simple Identification System for Rapidly Growing <i>Mycobacteria</i> (A Note)	
García Sabater, J. F., Alcon Limorte, J. J., Ferrer, M. A.	71
Biotyping of <i>Shigella sonnei</i> (A Note)	
Vlajinac, H., Kraljinić, S.	77

NUMBER 2

Antibiotics Effects on Growth and Heterocyst Differentiation of the Cyanobacterium <i>Nostoc muscorum</i>	
Pattanaik, U., Singh, P. K.	81
Selective Quantitative Determination of Tobramycin from Fermentation Broth	
Kiss, Gy. B., Széll, V., Ambrus, G., Ott, I.	89
Comparative Effects of Synthetic Insecticides — Endosulfan, Phosalone and Permethrin — on <i>Chlamydomonas reinhardtii</i> Algal Cells	
Gandhi, S. R., Kulkarni, S. B., Netravali, M. S.	93
Utilization of Organic Substrates by two Filamentous Cyanobacteria Under Various Growth Conditions	
Adhikary, S. P., Sahu J. K.	101
Effects of Hormones on the Multiplication of the Heterotrichous Protozoon <i>Blepharisma undulans</i> Stein	
Kovács, P., Csaba, G.	107
Determination of Phage Type, Colicin Type and Plasmid Profile in <i>Shigella sonnei</i>	
Tóth, I., Hajnal, A.	115

TENTH CONGRESS OF THE HUNGARIAN SOCIETY OF MICROBIOLOGY

Symposium I. Biotechnology and Industrial Microbiology	123
Symposium II. Biology of Interferon System	134
Symposium III. Latent and Persistent Viral Infections	148
Agricultural and Food Microbiology	151
Bacteriology and Parasitology	170
Immunology	192
Mycology	199
Virology	207

NUMBER 3

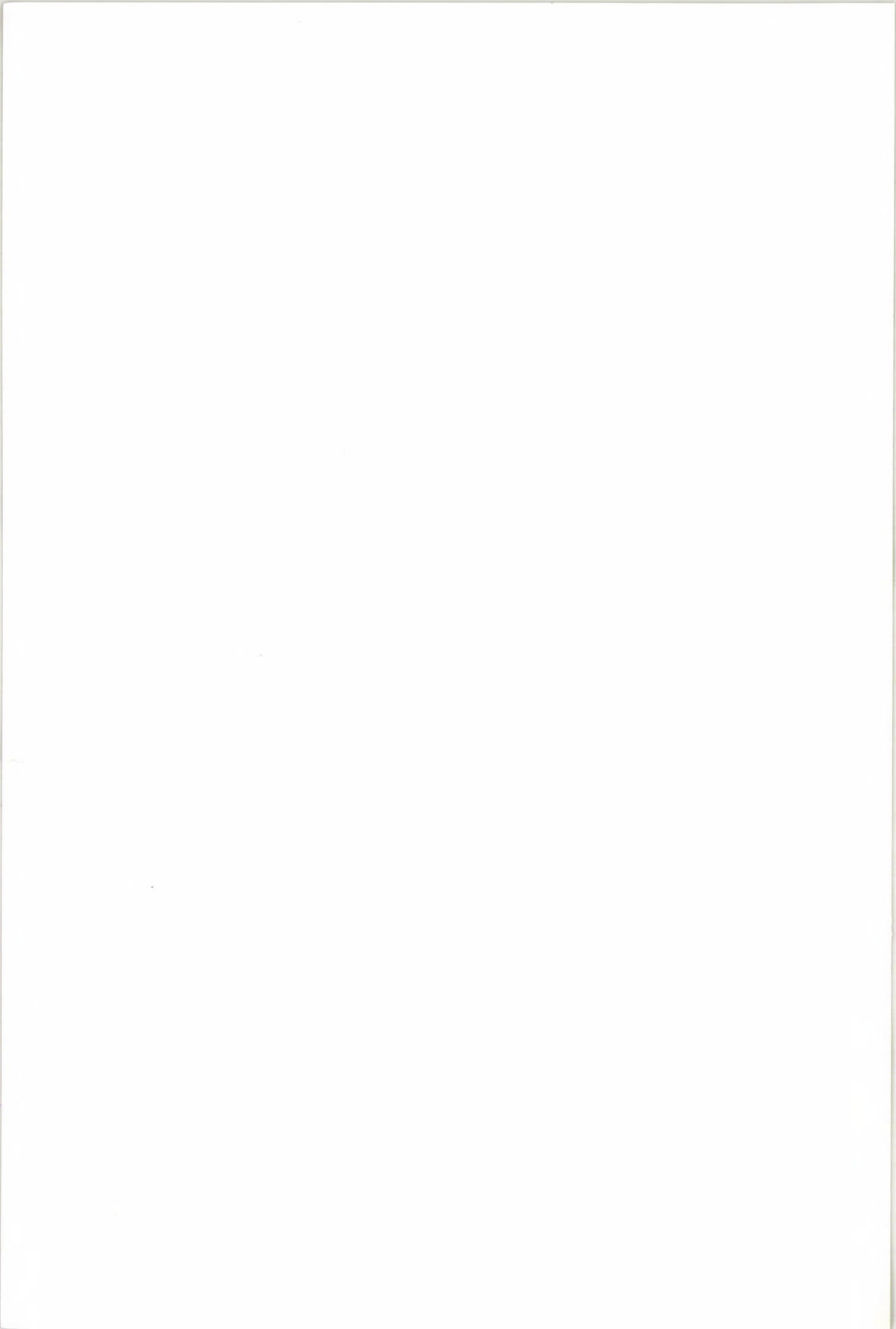
Phosphorus/Phosphonic Acid-Containing Antimicrobial Antibiotics (A Review)	
Uri, J. V., Bendas, C. M., Burdash, N.	221
The Kinetics of Cell Division in a Synchronizing Fermentor	
Novák, B., László, E.	259
Changes of Alternative Respiration During the Division Cycle of <i>Candida tropicalis</i>	
Novák, B., László, E.	269
Ring-Forming, Oligotrophic Microcycilus Organisms in the Water and Mud of Lake Balaton	
Langó, Zs.	277
Cell-Mediated Immune Response of Goats to Leptospirosis	
Siddique, I. H., Mohamed, A. A.	283
Subdivision of Common <i>Salmonella</i> Serotypes: Phage Typing of <i>S. virchow</i> , <i>S. manhattan</i> , <i>S. thompson</i> , <i>S. oranienburg</i> and <i>S. bareilly</i>	
G. László, V., Sz. Csórián, E.	289
Interferon Production in Myelo- and Lymphoproliferative Diseases. I. Spontaneous Interferon Production in Acute and Chronic Leukaemias	
Szabó, B., D. Tóth, F., Kiss, J., Vácsi, L., Kiss, A., Rák, K.	295
Alterations in the Chemical Composition and Antigenicity of <i>Escherichia coli</i> O89 Lipopolysaccharide and Lipid A after ⁶⁰ Co Gamma Irradiation	
Elekes, E., Lüderitz, O., Galanos, Ch., Bertók, L.	301
Virulence Associated Plasmid and R-Plasmid Curing in <i>Yersinia enterocolitica</i> by some Tricyclic Compounds	
Molnár, J., Nakamura, M. J.	307
Epidemiological Patterns and Incidence of Bio-, Sero- and Phage Types of <i>Vibrio cholerae</i> in Hyderabad, India, During 1971-1984	
Rathna, K., Khairunnisa, Rajyalakshmi, K., Naidu, A. S.	313
Priming of <i>Staphylococcus aureus</i> -Induced Interferon Production in Human Buffy Coat Leukocytes by Human Interferon-Alpha Pretreatment	
Rosztóczy, I.	323

NUMBER 4

The Aminothiazol-Oxyimino Cephalosporin Antibiotics with Quaternary Ammonium Substituents at the 3-Position (A Review)	
Uri, J. V., Burdash, N., Bendas, C. M.	327
Therapy of AIDS Patients — Classical Strategies and Proposals for Novel Therapeutic Approaches (A Short Review)	
Minárovits, J., Berencsi, G., Nász, I., Földes, I.	361
Production of Amylolytic Enzymes in Culture by <i>Botryodiplodia theobromae</i> and <i>Sclerotium rolfsii</i> Associated with the Corm Rots of <i>Colocasia esculenta</i>	
Nwufe, M. I., Fajola, A. O.	371
<i>Staphylococcus aureus</i> and <i>Staphylococcus epidermidis</i> Strains Differ in Interleukin 2 Inducing Activity	
Nemes Nagy, Zs., Rosztóczy, I.	379
Long-Term Preservation of the Enteroinvasive <i>Escherichia coli</i> Factor Responsible for Experimental Keratoconjunctivitis	
Sourek, J., Aldová, E.	383
Feeding by Mucin and Intestinal Growth of Some Enteric Bacterial Pathogens	
Kétyi, I.	389
Properties of Interferons Produced by Different Cell Populations	
Nossik, N. N., Bopegamage, S. A., Yershov, F. I.	397
Effects of Interferon and Tumour Necrosis Factor on Development of Intracellular Bacterial Infections	
Degré, M., Bukholm, G.	405
Inhibited Interferon Production After Space Flight	
Sonnenfeld, G., Gould, C. L., Williams, J. A., Mandel, A. D.	411
Antigenic Structure of Mammalian Adenoviruses Studied by Monoclonal Antibodies Against Bovine Adenovirus Type 2	
Rusvai, M., Belák, S., Stolz, B., Adám, Y., Nász, I.	417
Characterization of Multiply Resistant <i>Proteus mirabilis</i> Isolates in Hungary	
Konkoly Thege, M., Nikolnikov, S.	423
Effect of Herbicides on Growth of Some Microscopic Soil Fungus Species (A Note)	
Helmeczi, B., Kátai, J., Bessenyei, M.	429

CONTENTS

The Structure of O-Specific Polysaccharides and Serological Classification of <i>Pseudomonas aeruginosa</i> (A Review) Knirel, Yu. A., Vinogradov, E. V., Kocharova, N. A., Paramonov, N. A., Kochetkov, N. K., Dmitriev, B. A., Stanislavsky, E. S., Lányi, B.	3
Cytotoxic Activity of Lymphocyte Subpopulations Against Autologous Tumour Cells in Patients with Myeloid Leukaemias and Preleukaemic Disorders Szabó, B., Váczi, L., D. Tóth, F., Kiss, J., Kiss, A., Rák, K.	25
Production of Toxic Shock Syndrome Toxin-1 (TSST-1) Associated with <i>Staphylococcus aureus</i> From a Pyogenic Skin Infection Naidu, A. S., Rathna, K., Naidu, S., Rao, P. N., Rajyalakshmi, K.	35
Imprinting by Non-Signal Protein Molecule in <i>Tetrahymena</i> László, V., Csaba, G., Vargha, P.	41
Enhancement of Cilia Regeneration by Hormone Treatment of <i>Tetrahymena</i> Darvas, Zs., Árva, G., Csaba, G., Vargha, P.	45
Bacterial Translocation in Dianhydrotolcitol-Treated Mice Anderlik, P., Szeri, I., Bános, Zs.	49
A Phage Typing System for <i>Salmonella infantis</i> G. László, V., Csák, K., Sz. Csórián, E.	55
A Simple Identification System for Rapidly Growing Mycobacteria (A Note) García Sabater, J. F., Alcon Limorte, J. J., Ferrer, M. A.	71
Biotyping of <i>Shigella sonnei</i> (A Note) Vlajinac, H., Krajinović, S.	77



THE STRUCTURE OF O-SPECIFIC POLYSACCHARIDES AND SEROLOGICAL CLASSIFICATION OF *PSEUDOMONAS AERUGINOSA*

(A REVIEW)

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Introduction	3
Monosaccharide composition of O-specific polysaccharides	5
Structures of O-specific polysaccharides	8
Discussion	21
Conclusion	22
References	23

Introduction

On the basis of serological specificity of O antigens, several serotyping schemes have been proposed for *Pseudomonas aeruginosa*. The number of O serogroups and subgroups and their correspondence in the different serogrouping systems have been reviewed by Lányi and Bergan [1] and Stanislavsky et al. [2]. In an attempt to establish a unified scheme, Lányi and Bergan [1] recommended to designate O serogroups according to the Habs scheme [3], but to subdivide them into subgroups as described by Lányi [4] and to supplement with the antigens not included in these two systems (Sandvik [5], Verder and Evans [6], Meitert [7]). The Habs O2 and O5 serogroups, as well as the O7 and O8 serogroups, have been pairwise united into the O2 and O7

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serogroups, respectively, on the basis that they share well-defined partial antigens. This compiled scheme was later supported by Akatova and Smirnova [8] and Homma [9], and the former authors have supplemented it with new subgroups represented by strains chosen from Wokatsch's collection [10].

On the basis of challenge protection in mice Fisher et al. [11] have defined seven groups of cross-protective homogeneity (immunotypes 1-7). A heptavalent vaccine comprising a mixture of the lipopolysaccharides from strains representative of the seven immunotypes has been developed and used for the prophylaxis of *P. aeruginosa* infections [12]. The O antigens of the immunotypes have been defined according to various antigenic schemes [1]. Table I shows the correspondence of O antigens in some of the schemes.

Table I
Antigenic schemes for P. aeruginosa discussed in this review
Cited from [2]

Compiled scheme			Original schemes		
Lányi and Bergan [1]			Habs [3]	Lányi [4]	Fisher [11]
Serogroup	Partial O antigens	Reference strains	O antigens		Immuno- type
1	1	170014/Habs 1	1	6	4
2	2a,2b	170003	—	3a,3b	—
	2a,2b,2c*	Wokatsch 25	—	—	—
	(2a),2c	170004/Habs 2	2	(3a),3c	3
	2a,2d	170005	5	3a,3d	7
	2a,2d,2e	170006	—	3a,3d,3e	—
	(2a),2d,2f	170007	—	(3a),3d,3f	—
3	3a,3b*	Wokatsch 14	—	—	—
	3a,3b,3c*	170001/Habs 3	3	1	—
	3a,3d*	Wokatsch 13	—	—	—
4	4a,4b	170021/Habs 4	4	11	—
	4a,4c	170040	—	—	—
6	6a, . . .	Habs 6	6	4a, . . .	1
	6a,6b	170008	—	4a,4b	—
	6a,6c	170009	—	4a,4c	—
	6a,6d	170010	—	4a,4d	—
7	7a,7b,7c	170011	7	5a,5b,5c	—
	7a,7b,7d	170012	8	5a,5b,5d	—
	7a,7d	170013	—	5a,5d	6
9	9a,9b,9d*	170020	—	10a,10b	—
	9a,9c*	Wokatsch 16	—	—	—
	9a,9d*	170019	9	10a	—
10	10a,10b	Habs 10	10	—	—
	10a,10c	170002	—	2	5
11	11a,11b	170015/Habs 11	11	7a,7b	2
	11a,11c	170016	—	7a,7c	—
12	12	170023/Habs 12	12	13	—
13	13a,13b	Sandvik II	—	—	—
	13a,13c	Verder-Evans 1M-1	—	—	—
14	14	Meitert X	—	—	—
15	15	170022	—	12	—

* Supplemented by Akatova and Smirnova [8].

Since the late 1960s many attempts have been undertaken to correlate immunospecificity of *P. aeruginosa* strains with structures of O antigenic lipopolysaccharides. They have been found to possess the same general architecture as the most thoroughly studied lipopolysaccharides from enterobacteria. The *P. aeruginosa* lipopolysaccharides contain a hydrophobic lipid part (lipid A), to which an O-specific polysaccharide chain is attached via a common core oligosaccharide (see Review [13]). The structurally most conservative part of the molecule, lipid A, being similar, on the whole, to lipid A of enterobacteria, has some points of difference, which include the fatty acid composition and the unusually high degree of phosphorylation [13]. The core region, together with the components common to core oligosaccharides from enterobacteria (D-glucose, L-glycero-D-manno-heptose, 3-deoxy-D-manno-octulosonic acid, phosphate, possibly ethanolamine), contains L-rhamnose, D-galactosamine, and L-alanine. Partial structures have been proposed for the core oligosaccharides of lipopolysaccharides from strains NCTC 1999 [14] and PAC1R [15].

The most variable part of the lipopolysaccharide molecule is an O-specific polysaccharide chain, which the serotype-specific determinants are associated with. Analysis of the monosaccharide composition of the O-specific polysaccharides, that was carried out in the 1970s [16–23], revealed the presence of xylose, glucose, rhamnose, glucosamine, 2,4-diamino-2,4,6-trideoxy-D-glucose, and L-galactosaminuronic acid. However, many other amino components failed to be identified, and this has delayed the progress in the structural determination of the polysaccharides until the 1980s. Some data on the structures of the *P. aeruginosa* O-specific polysaccharides are available in recent reviews [1, 13, 24], these are still far from being complete since O antigens of many serogroups have been studied in detail only in the last 3–4 years.

The present paper summarizes the data on the composition and structures of the O-specific polysaccharide chains of lipopolysaccharides from *P. aeruginosa* strains representative of all the O serogroups in the Lányi–Bergan classification scheme [1] supplemented by Akatova and Smirnova [8] as well as of the seven Fisher immunotypes [11].

Monosaccharide composition of O-specific polysaccharides*

The composition of *P. aeruginosa* O-specific polysaccharides (Table II) differs essentially from that of polysaccharides of many other Gram-negative bacteria studied [25]. First of all, *P. aeruginosa* polysaccharides infrequently

* *Abbreviations.* QuiN, 2-amino-2,6-dideoxyglucose (quinovosamine); FucN, 2-amino-2,6-dideoxygalactose (fucosamine); Qui4N, 4-amino-4,6-dideoxyglucose; Bac(2N4N), Bac(NAc)₂, 2,4-diamino-, 2,4-diacetamido-2,4,6-trideoxyglucose (bacillosamine, di-N-acetyl-

contain neutral sugars, in which the latter are usually abundant. Of four neutral sugars found in *P. aeruginosa* O polysaccharides (D-glucose, L-rhamnose, D-xylose, D-ribose) only rhamnose is encountered frequently (in six out of the thirteen serogroups), and the polysaccharides of four serogroups do not contain any neutral sugars.

The *P. aeruginosa* polysaccharides are typically rich in monoamino and diamino sugars (Fig. 1), many of them carrying simultaneously a carboxyl function. The polysaccharides fail to contain acidic components in only four serogroups. Among amino sugars widely distributed in nature, hexosamines (D-glucosamine, D-galactosamine) are encountered rather infrequently, whereas their far less common 6-deoxy derivatives (D-quinovosamine, D- and L-fucosamine) are present in most polysaccharides. Some derivatives (formulae 1 and 2 in Fig. 1) of a diamino sugar, bacillosamine (2,4-diamino-2,4,6-trideoxyglucose) have also been found [22, 26].

Acidic monosaccharides are represented by three classes: 2-amino-2-deoxyuronic acids (derivatives of D- and L-galacto isomers 3-7) [22, 27-30], 2,3-diamino-2,3-dideoxyuronic acids (derivatives of D-glucosamine, D-mannosamine, L-gulonic isomers 8-12) [31-36], and 5,7-diamino-3,5,7,9-tetradeoxynonulosonic acids (derivatives of L-glycero-L-manno isomer, pseudominic acid 13-15, and D-glycero-L-galacto isomer 16) [37-40]. The last two classes of monosaccharides have been found for the first time in nature as constituents of *P. aeruginosa* polysaccharides. New ulosonic acids are similar in structure to the well-known neuraminic acid (5-amino-3,5-dideoxy-D-glycero-D-galacto-nonulosonic acid) [41], but differ from it by the presence of an additional amino group at C-7, of a deoxy unit at C-9, and by the configuration.

Most amino groups of the amino sugars are acetylated, but in some cases they carry acyl substituents that occur rarely in natural carbohydrates, such as formyl group (derivatives of D-galactosaminuronic acid 5 and 7, of pseudaminic acid 14 and 15), (R)- and (S)-3-hydroxybutyryl groups (derivatives of bacillosamine 2 and pseudaminic acid 13 and 15), and acetimidoyl group (derivatives of 2,3-diamino-2,3-dideoxyuronic acids 11 and 12, and of L-fucosamine 17). The acetimidoyl group has not been found in any other natural source. It is of tautomeric character (formulae 11, 12 and 17 show one of the possible tautomers) and endows the corresponding monosaccharides with basic or, in case of uronic acid derivatives, amphoteric properties.

bacillosamine), respectively; GalNAcA, GalNFmA, 2-acetamido-, 2-formamido-2-deoxygalacturonic acid (N-acetyl-, N-formylgalactosaminuronic acid), respectively; Glc(2N3N)A, Glc(NAc)₂A, Man(2N3N)A, Man(NAc)₂A, Gul(2N3N)A, Gul(NAc)₂A, 2,3-diamino-, 2,3-diacetamido-2,3-dideoxygluc-, mann-, guluronic acid, respectively; Pse(5N7N), Pse(5NAc7NFm), 5,7-diamino-, 5-acetamido-7-formamido-3,5,7,9-tetradeoxy-L-glycero-L-manno-nonulosonic acid (pseudaminic acid, 5,N-acetyl-7-N-formylpseudaminic acid), respectively; Non(5N7N), Non(NAc)₂, 5,7-diamino-, 5,7-diacetamido-3,5,7,9-tetradeoxy-D-glycero-L-galacto-nonulosonic acid, respectively.

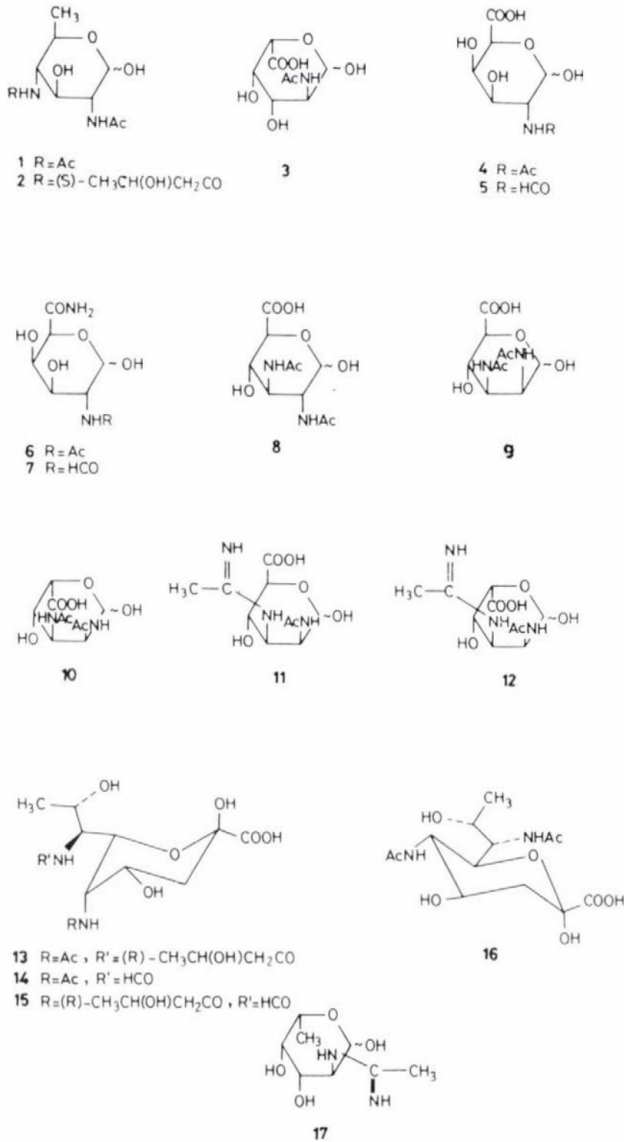


Fig. 1. Monosaccharide constituents of *P. aeruginosa* polysaccharides

N-Acyl derivatives of D-galactosaminuronic acid are found both as free acids 4 and 5, and as primary amides 6 and 7. Finally, many constituent monosaccharides (rhamnose, *N*-acetyl-D-fucosamine, derivatives of D- and L-galactosaminuronic acid 3–7 and of pseudaminic acid 13) carry *O*-acetyl groups, which have been found in 7 serogroups.

Table II

Distribution of monosaccharides and non-carbohydrate components in *P. aeruginosa* O-specific polysaccharides

Components	O-Serogroup														
	1	2	3	4	6	7	9	10	11	12	13	14	15		
Monosaccharides															
DRib														+	
DXyl						+									
DGlc									+		+				
LRha			+	+	+			+			+	+			
DGlcN			+												
DGalN	+														
DManN														+	
DQuiN	+			+	+		+	+		+	+				
DFucN	+	+		+		+	+		+		+				
LFucN				+					+	+					
DBac(2N4N)			+												
DGalNA					+						+				
LGalNA			+					+							
DGlc(2N3N)A	+														
DMan(2N3N)A		+													
LGul(2N3N)A		+													
Pse(5N7N)						+	+								
Non(5N7N)										+					
N-Substituents															
Ac	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
HCO					+	+									
(R)-CH ₃ CH(OH)CH ₂ CO						+	+								
(S)-CH ₃ CH(OH)CH ₂ CO			+				+								
NH=C(CH ₃)		+									+				
O-Substituents															
Ac		+	+		+	+	+	+			+				
Carboxyl substituents															
NH ₂					+										

Data in Table II show that the O-specific polysaccharide chains of lipopolysaccharides of various O serogroups differ in their composition, and, hence, O-specificity of the respective strains correlates with the monosaccharide composition of O antigens.

Structures of O-specific polysaccharides

Serogroup O1

The O1 serogroup is not subdivided into subgroups. The O-specific structure of the reference strain 170014 is an acidic polysaccharide [42]. It is built up exclusively of N-acetylated amino sugars, the acidic component

being 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid which is the first representative of this class of monosaccharides found in nature [31, 32].

18 -4)- β -D-Glc(NAc)₂A-(1-3)- α -D-FucNAc-(1-3)- α -D-QuiNAc-(1-4)- α -D-GalNAc-(1-Serogroup O1, strains 170014, Habs 1, Fisher 4

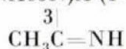
The polysaccharide having the same structure as strain 170014 is characteristic also of the reference strains of Habs serogroup O1 and Fisher immunotype 4 [43]; this finding is consistent with the serological similarity between these strains [1]. The identification of 2,3-diamino-2,3-dideoxy-D-glucuronic acid in the polysaccharide of *P. aeruginosa* P14 and of some other strains, also belonging to serogroup O1, suggests that their O-specific polysaccharides have structure **18** as well.

Serogroup O2

This complex serogroup includes six subgroups, whose O-specificity is determined by polysaccharides with structures **19–25** [44–46]. The trisaccharide repeating units of these polysaccharides have the same general structures and consist of the residues of 2-acetamido-3-acetamidino- and 2,3-diacetamido-derivatives of uronic acids having the β -D-manno or α -L-gulo configuration, and of N-acetyl-D-fucosamine. Some of the O2 polysaccharides lack a strictly regular structure. Thus, the O(2a),2d,2f polysaccharide involves in comparable amounts the trisaccharide units of two types **24** and **25**, which differ from each other in configuration at C-5 of 2-acetamido-3-acetamidino-derivative of uronic acid. Two other polysaccharides, containing a derivative of α -L-guluronic acid, namely those of subgroups O(2a),2c and O2a,2d,2c, along with the main repeating units of structures **21** and **23**, involve a small proportion (cca. 10%) of units, in which 2,3-diacetamido-2,3-dideoxy- α -L-guluronic acid is replaced by the β -D-manno isomer, i.e. of the units of structures **19** and **22**, typical of subgroups O2a,2b and O2a,2d, respectively.

Serological differentiation of the strains within the O2 serogroup is related to the structural features of the O-specific polysaccharides. Among these features are the presence or absence of O-acetyl groups, the anomerid configuration of N-acetylglucosamine residue, and the configuration at C-5 or the uronic acid derivatives. The variation of the C-5 configuration interconverts the β -D-manno isomer and the α -L-gulo isomer and, hence, it is related to a change in the monosaccharide composition of the polysaccharides.

19 -4)- β -D-Man(2NAc3N)A-(1-4)- β -D-Man(NAc)₂A-(1-3)- β -D-FucNAc-(1-



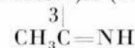
Subgroup O2a,2b, strain 170003

- 20** -4)- β -D-Man(2NAc3N)A-(1-4)- β -D-Man(NAc)₂A-(1-3)- β -D-FucNAc-(1-



Subgroup O2a,2b,2c, strains Wokatsch 25, PAO-D3

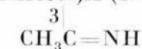
- 21** -4)- β -D-Man(2NAc3N)A-(1-4)- α -L-Gul(NAc)₂A-(1-3)- β -D-FucNAc-(1-



Subgroup O(2a),2c, strains 170004, Habs 3, Fisher 3

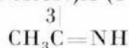
Besides the major units of structure **21**, a small proportion (cca. 10%) of units of structure **19** is present in the polysaccharide.

- 22** -4)- β -D-Man(2NAc3N)A-(1-4)- β -D-Man(NAc)₂A-(1-3)- α -D-FucNAc-(1-



Subgroup O2a,2d, strains 170005, Habs 5, PaO1

- 23** -4)- β -D-Man(2NAc3N)A-(1-4)- α -L-Gul(NAc)₂A-(1-3)- α -D-FucNAc-(1-



Subgroup O2a,2d,2e, strain 170006

Besides the major units of structure **23**, a small proportion (cca. 10%) of units of structure **22** is present in the polysaccharide.

- 24** -4)- α -L-Gul(2NAc3N)A-(1-4)- β -D-Man(NAc)₂A-(1-3)- α -D-FucNAc-(1-



- 25** -4)- β -D-Man(2NAc3N)A-(1-4)- β -D-Man(NAc)₂A-(1-3)- α -D-FucNAc-(1-



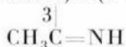
Subgroup O(2a),2d,2f, strain 170007

The proportion of structures **24** and **25** in the polysaccharide is cca 2:1.

All polysaccharides of this group contain a common monosaccharide component, 2-acetamido-3-acetamidino-2,3-dideoxy-D-mannuronic acid, which seems to determine the group O factor 2a. Among other factors, common to several subgroups, the O factor 2b, characteristic of the O2a,2b and O2a,2b,2c subgroups, is probably related to a fragment of the carbohydrate skeleton, which is the same for both polysaccharides (structures **19** and **20**). The O factor 2d, common to subgroups O2a,2d, O2a,2d,2e, and O(2a),2d,2f, may be due to the presence of the trisaccharide units of structure **22**; for the O2a,2d subgroup these are the only type of units, for the O2a,2d,2e subgroup they represent the minor type of units, and for the O(2a),2d,2f subgroup they account for cca. 35% of the total trisaccharide units. The presence in the O-specific polysaccharide of two types of trisaccharide units may also account for the fact that some strains, belonging to serogroup O2, react in both anti-O2b and anti-O2c sera [47], although no subgroup has been established which contains simultaneously O2b and O2c antigens. In fact, the O(2a),2c polysaccharide contains both the units of structure **21** (major type), which seems to be related to the factor O2c, and the units of structure **19** (minor type),

determining the factor O2b. As for the O factor 2e typical of subgroups O2a,2b,2e and O2a,2d,2e, it is difficult to relate it unambiguously to any common fragment in the corresponding polysaccharides (structures **20** and **23**).

26 -4)- α -L-Gul(2NAc3N)A-(1-4)- β -D-Man(NAc)₂A-(1-3)- α -D-FucNAc-(1-



Strain Fisher 7

Besides the major units of structure **26**, a small proportion (cca. 10%) of units of structure **22** is present in the polysaccharide.

As it may be anticipated on the basis of the serological data [1], the polysaccharides of the strains representing the Habs O2 and O5 serogroups have the structures **21** and **22**, respectively. The Fisher immunotype 3 polysaccharide has the structure **21**, which is consistent with its serological similarity to subgroup O(2a),2c [1]. However, no such correlation is observed for the Fisher immunotype 7, which according to serological classification [1] falls in subgroup O2a,2d. The Fisher 7 polysaccharide is built up mainly of the trisaccharide units of structure **26** [46], which is not identical with any of the O2 polysaccharide structures. Like the polysaccharides of serogroup O2, containing derivatives of L-guluronic acid, the Fisher 7 polysaccharide involves a small proportion (cca. 10%) of the trisaccharide units of structure **22**. The presence of these minor units seems to determine the factors O2a and O2d, and as a consequence, the cross-reaction between Fisher 7 and subgroup O2a,2d. It may also be assumed that Fisher 7 along with the factors O2a and O2d has another O factor, also present in subgroup O(2a),2d,2f, since the major trisaccharide units of their polysaccharides (structures **26** and **24**) have an identical carbohydrate skeleton.

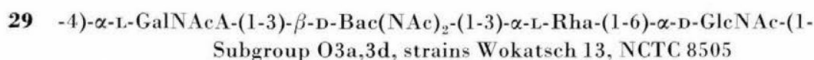
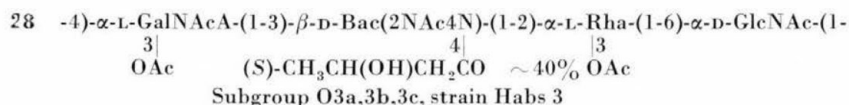
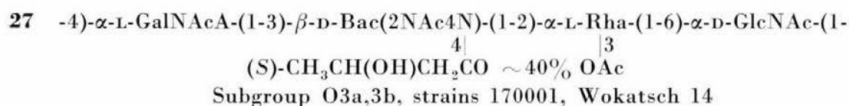
Accordingly, on the basis of carbohydrate structure, it seems that Fisher 7 represents an individual, seventh subgroup of serogroup O2. This assumption is supported by cross-absorption experiments showing that the O antigens in Fisher 7 and in 170005 are not identical (Lányi, unpublished observation). In view of the above data, further investigation is required to determine the complete set of partial antigens in Fisher 7 and to define a procedure for preparing an absorbed serum for this so far undefined O factor.

A peculiar change in the structure of the O-specific polysaccharide was observed on lysogenization of *P. aeruginosa* strain PAO1 by bacteriophage D3 [48]. This strain, having structure **22**, belongs to subgroup O2a,2d [48]. The polysaccharide of the lysogenized strain PAO-D3 has structure **20** which is typical of the O2a,2b,2e subgroup, and thus, the phage conversion resulted in the inversion of the anomeric configuration of the N-acetylglucosamine residue and the introduction of an O-acetyl group into position 4 of this sugar. Such a conversion allows to suggest that the observed diversity of the structures of the O2 polysaccharides as well as, perhaps, of polysaccharides

of other *P. aeruginosa* serogroups, results from an alteration of a parent polysaccharide (polysaccharides) by bacteriophages in the course of the evolution of the microorganism.

Serogroup O3

This serogroup has been subdivided by Akatova and Smirnova [8] into subgroups O3a,3b, O3a,3b,3c, and O3a,3d. The O-specific polysaccharides have structures **27–29** [26, 49], are acidic and are composed of identical monosaccharides. The tetrasaccharide repeating units contain the residues of L-rhamnose, D-glucosamine, D-bacillosamine, and L-galactosaminuronic acid. In addition, the polysaccharides possess an identical sequence of the monosaccharide residues, identical configurations of all glycosidic linkages and identical modes of substitution of the residues of amino sugars. The differences between the polysaccharides of this group are associated with the presence or absence of O-acetyl group at some of the rhamnose residues (degree of O-acetylation cca. 40%) and at the residues of N-acetyl galactosaminuronic acid (degree of O-acetylation nearly stoichiometric), with variation of the acyl substituent at N-4 of the bacillosamine residue [acetyl or (S)-3-hydroxybutyryl group], as well as with the mode of substitution of the rhamnose residue (at position 2 or 3).



Comparison of the structures of these three O-specific polysaccharides allows to determine tentatively the epitopes of the O factors responsible for the serological similarity and differentiation within the O3 serogroup. All the polysaccharides contain a trisaccharide fragment with identical carbohydrate skeleton, and this fragment, or part of it, determines the group O factor 3a. The O factor 3b, common to subgroups O3a,3b and O3a,3b,3c, is related to the 4-N-(3-hydroxybutyryl) derivative of bacillosamine (structures **27** and **28**), whereas the O factor 3d, missing in these two subgroups but present in the O3a,3d subgroup, is associated with the di-N-acetyl derivative of bacillosamine (structure **29**). Finally, the O factor 3c, characteristic of the O3a,3b,3c

subgroup, is due to the *O*-acetyl group at position 3 of the residues of *N*-acetylgalactosaminuronic acid (structure **28**), which is absent from the polysaccharides of other groups.

In this serogroup there is a discrepancy between serological findings and polysaccharide structures. Serologically, strains 170001 and Habs 3 had been regarded as identical on the basis of cross-absorption tests [1] and later both strains were classified as having partial antigens O3a,3b,3c [8]. However, the structures of the *O*-specific polysaccharides of strains 170001 and Habs 3 turned out to be distinct. In contrast, strain Wokatsch 14, classified into subgroup O3a,3b [8], has the same polysaccharide structure as strain 170001. Strain Wokatsch 13 differs in polysaccharide structure from the above strains and, having the distinct subgroup antigen O3d [8], may be classified as representative of a separate subgroup. *P. aeruginosa* strain NCTC 8505 is identical in polysaccharide structure with Wokatsch 13. The results indicate that classification of serogroup O3 into subgroups still awaits elucidation.

Serogroup O4

Serogroup O4 includes two subgroups, O4a,4b and O4a,4c. The *O*-specific polysaccharides of their reference strains (170021 and 170040) have structures **30** and **31** [50]. These polysaccharides are built up of tetrasaccharide repeating units involving a residue of *L*-rhamnose, two residues of *N*-acetyl-*L*-fucosamine and one residue of *N*-acetyl-6-deoxy-*D*-hexosamine, which has the *gluco* configuration in the O4a,4b subgroup or *galacto* configuration in the O4a,4c subgroup (i.e. *D*-quinovosamine or *D*-fucosamine, respectively). The sequence, the anomeric configuration and modes of substitution of the monosaccharide residues are identical in both polysaccharides, and, hence, the configuration at C-4 of the 6-deoxy-*D*-hexosamine residue is the only distinction between the *O*-antigens.

30 -2)- α -*L*-Rha-(1-3)- α -*L*-FucNAc-(1-3)- α -*L*-FucNAc-(1-3)- β -*D*-QuiNAc-(1-Subgroup O4a,4b, strains 170021, Habs 4

31 -2)- α -*L*-Rha-(1-3)- α -*L*-FucNAc-(1-3)- α -FucNAc-(1-3)- β -*D*-FucNAc-(1-Subgroup O4a,4c, strain 170040

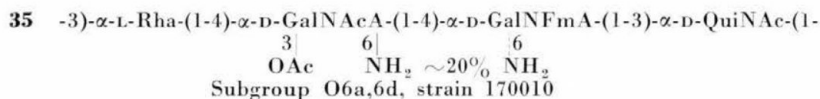
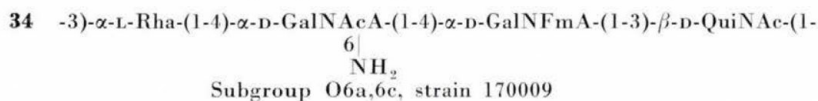
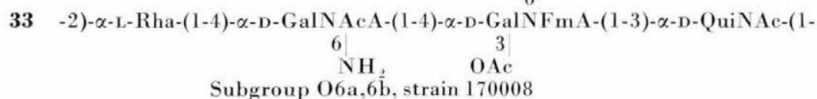
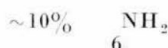
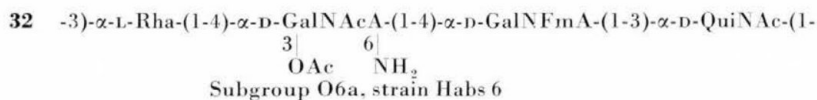
The group O factor 4a seems to be associated with the common trisaccharide fragment built up to the *L*-rhamnose and two *N*-acetyl-*L*-fucosamine residues. The O factors 4b and 4c are related to the *N*-acetyl-*D*-quinovosamine residue or to the *N*-acetyl-*D*-fucosamine residue, respectively. Consistent with their serological identity, the Habs 4 polysaccharide has the same structure as that of strain 170021.

Serogroup O5

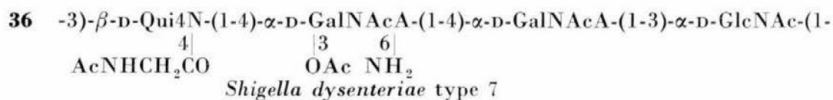
Habs' serogroup O5 corresponds to a subgroup of serogroup O2, and has therefore been omitted from the compiled scheme [1].

Serogroup O6

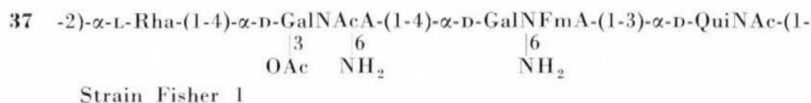
This serogroup is subdivided into four subgroups, whose O-specific polysaccharides have structures **32–35** [51, 52]. All of these polysaccharides have an identical monosaccharide composition, and are built up of tetrasaccharide repeating units, containing the residues of L-rhamnose, *N*-acetyl-D-quinovosamine, *N*-formyl-D-galactosaminuronic acid or *N*-formyl-D-galactosaminuronamide, and *N*-acetyl-D-galactosaminuronamide. All of them but the O6a,6c polysaccharide also contain *O*-acetyl groups (~ 0.8 per one repeating unit).



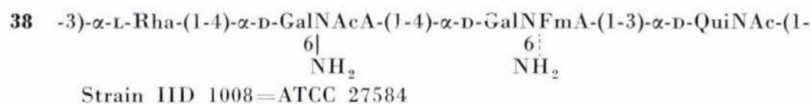
These polysaccharides possess the same sequence of monosaccharide residues, the same modes of substitution of the three amino sugars, and the same anomeric configurations of the residues of rhamnose and derivatives of galactosaminuronic acid. As a consequence, they contain a common oligosaccharide fragment, involving both derivatives of galactosaminuronic acid, which seems to determine the group O factor 6a. It is noteworthy that a similar fragment is present in the O-specific polysaccharide of *Shigella dysenteriae* type 7 (structure **36**) [53], and this fact evidently accounts for the serological similarity between this microorganism and *P. aeruginosa* O6 strains [54].



The O6 polysaccharides differ from each other by the mode of substitution of the rhamnose residue (at position 2 or 3), by the anomeric configuration of the *N*-acetylquinovosamine residue, as well as by the presence or absence and the position of the *O*-acetyl group (at C-3 of one of the derivatives of galactosaminuronic acid) and by the partial amidation of *N*-formylgalactosaminuronic acid. The O factors 6b, 6c and 6d are undoubtedly related to some of these distinctions, but it is difficult to determine unambiguously their epitopes unless an additional immunochemical investigation is performed.



Comparison of the Fisher immunotype 1 polysaccharide (structure 37) [51, 52] with those of serogroup O6 reference strains (structures 32–35) showed that it is similar to, but not identical with, any of the O6 polysaccharides. The former resembles most closely the O6a,6c polysaccharide, both having the identical carbohydrate skeleton but differing in the position of *O*-acetyl group and in the degree of amidation of *N*-formylgalactosaminuronic acid (in the Fisher 1 polysaccharide all residues of uronic acids are amidated). This finding supports an earlier observation (Lányi, unpublished data) that the antigen termed O6 is a complex factor: reference strains for subgroups O6a,6b, O6a,6c and O6a,6d (170008, 170009 and 170010, respectively) fail to remove all agglutinins from serum Habs 6. Accordingly, in addition to the group antigen O6a, Habs 6 must contain one or more partial antigens absent from the other subgroups. Likewise, Fisher 1 may bear additional antigens. Until further serological evidence is available on this point, it seems justified to classify Habs 6 and Fisher 1 into different subgroups both designated O6a, . . . (indicating that in addition to the group antigen, other factors may be present).



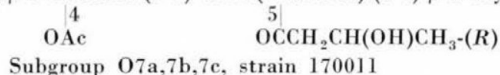
Analysis of *P. aeruginosa* IID 1008 (ATCC 27584), which is the reference strain for Homma serogroup G [55], showed that its O-specific polysaccharide had the same structure as the O6a,6c polysaccharide, with the exception that approximately a half of the total *N*-formylgalactosaminuronic acid residues exists in the form of the primary amide (structure **38**) [29].

Serogroup 07

The O7 serogroup includes three subgroups, O7a,7b,7c, O7a,7b,7d and O7a,7d. The O-specific polysaccharides of the reference strains are char-

acterized by structures **39–41** [56]. They are built up of trisaccharide repeating units involving the residues of D-xylose, N-acetyl-D-fucosamine, and di-N-acyl derivative of pseudaminic acid

39 -3)- β -D-FucNAc-(1-4)- α -Pse(5N7NFm)-(2-4)- β -D-Xyl-(1-



40 -3)- β -D-FucNAc-(1-4)- α -Pse(5NAc7NFm)-(2-4)- β -D-Xyl-(1-
(Subgroup O7a,7b,7d, strains 170012, Habs 8

41 -3)- β -D-FucNAc-(1-4)- α -Pse(5NAc7NFm)-(2-4)- β -D-Xyl-(1-
Subgroup O7a,7d, strains 170013, Fisher 6

All three polysaccharides have identical carbohydrate skeleton, which, evidently, determines the group O factor 7a. The difference between these polysaccharides is related to the presence or absence of the O-acetyl group at position 4 of the N-acetylfucosamine residue, and by the variation of N-acyl substituent at position 5 of pseudaminic acid [(R)-3-hydroxybutyryl or acetyl group]. The O-acetyl group seems to determine the O factor 7b, characteristic of subgroups O7a,7b,7c and O7a,7b,7d (structures **39** and **40**), whereas the O factor 7d in subgroups O7a,7b,7d and O7a,7d is associated with 5-N-acetyl-7-N-formylpseudaminic acid (structures **40** and **41**). The O factor 7c, characteristic of the O7a,7b,7c subgroup only, is related to 7-N-formyl-5-N-[(R)-3-hydroxybutyryl]-pseudaminic acid (structure **39**).

The O-specific polysaccharide of the Habs O8 serogroup is identical to that of the O7a,7b,7d subgroup. This finding is consistent with that of Lányi and Bergan [1], who classified strain Habs 8 into subgroup O7a,7b,7d. Unfortunately, in the review by Stanislavsky et al. [2], Habs serogroup O8 was misprinted as corresponding to subgroup O7a,7d.

As for the Habs O7 serogroup, we failed to isolate any O-specific polysaccharide from the reference strain, which seems, therefore, to produce R lipopolysaccharide. The Fisher immunotype 6 O-specific polysaccharide has structure **41** [56] that is in accordance with the serological identity of Fisher 6 and the reference strain for subgroup O7a,7d [1].

Serogroup O8

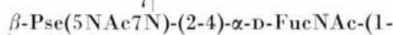
Habs' serogroup O8 corresponds to a subgroup of serogroup O7, and has therefore been omitted from the compiled scheme [1].

Serogroup O9

In the original scheme of Lányi [4] this serogroup is subdivided into two subgroups represented by strains 170020 and 170019. For these strains structures **42** and **43** have been established [57]. The polysaccharides are identical

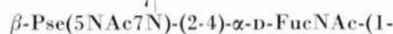
in monosaccharide composition and are built up of trisaccharide repeating units, containing the residues of *N*-acetyl-D-quinovosamine, *N*-acetyl-D-fucosamine, and 5-*N*-acetyl-7-*N*-[(*R*)-3-hydroxybutyryl] pseudaminic acid. A remarkable feature of these polysaccharides is that the *N*-acetylquinovosamine residue is attached to pseudaminic acid via the *N*-acyl substituent, i.e. via the 3-hydroxybutyryl group. They are, thus, biopolymers of a new type, in which monomers are connected not only by the glycosidic linkages, but also by the amidic linkages.

42 -3)-β-D-QuiNAc-(1-O



Subgroup O9a,9b, strain 170020

43 -3)-β-D-QuiNAc-(1-O



Subgroup O9a, strains 170019, Habs 9

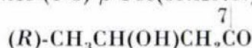
The O9 polysaccharides possess an identical carbohydrate skeleton which evidently, determines the O factor(s) shared by strains 170020 and 170019. The O factor 9b is associated with the *O*-acetyl group at position 4 of pseudaminic acid; the presence of this group is the only distinction between these two polysaccharides.

Serogroup O9 has been further subdivided by Akatova and Smirnova [8], who described a new partial antigen, O9d in strains 170020 and 170019, and established a new subgroup, O9a,9c represented by strain Wokatsch 16. However, as in the case of strain Habs 7, we failed to obtain any O-specific polysaccharide from Wokatsch 16. Thus, it remains unclear whether the factor O9c is determined by any fragment of the lipopolysaccharide or it is related to another heat-stable antigen. As serological definition of antigen 9d depends on the existence of subgroup O9a,9c, further investigations are needed in this respect.

The Habs serogroup O9 polysaccharide proved to be identical with that of strain 170019 (structure 43), in agreement with the serological identity of their O antigens [1].



44 -3)-α-D-GlcNAc-(1-8)-β-Pse(5NAc7N)-(2-6)-α-D-Gal-(1-6)-α-D-Glc-(1-2)-β-D-Galf-(1-

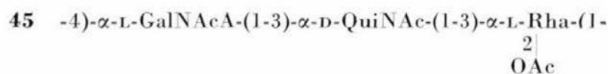


Shigella boydii type 7

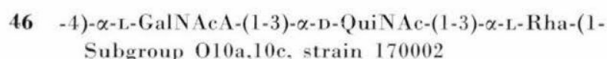
The serological similarity of *P. aeruginosa* O9 and *Shigella boydii* type 7 strains is known [54]. The O-specific polysaccharide of the latter has structure 44 [58] and contains the same derivative of pseudaminic acid as the O9 polysaccharides. This derivative is evidently responsible for a pronounced cross-reactivity between these two microorganisms. It is noteworthy that the immunodominant role of this sugar is not affected either by the absence of any common neighbouring monosaccharide or by the different mode of substitution (at the *N*-acyl substituent or at position 8).

Serogroup O10

Serogroup O10 includes subgroups O10a,10b and O10a,10c, whose O-specific polysaccharides have structures 45 and 46, respectively [27]. They are characterized by an identical monosaccharide composition and trisaccharide repeating units involving the residues of L-rhamnose, *N*-acyl-D-quinovosamine, and *N*-acetyl-L-galactosaminuronic acid. The polysaccharides also have identical carbohydrate skeletons and differ from each other by the presence or absence of the *O*-acetyl group at position 2 of the rhamnose residue (the degree of acetylation is $\sim 80\%$).



Subgroup O10a,10b, strains Habs 10, Fisher 5



The group O factor 10a is evidently related to a common fragment of the backbone (a disaccharide or, perhaps, a trisaccharide), the residue of 2-acetamido-2-deoxy-L-galacturonic acid being the immunodominant sugar, since the carboxyl-reduced O10a,10c polysaccharide lacks any serological activity. The O factor 10b is associated with the *O*-acetyl group, while the O factor 10c is related to the non-acetylated residue of rhamnose; this is confirmed by the fact that *O*-deacetylation of the O10a,10b polysaccharide results in a change of O-specificity due to the transition of the O factor 10b to the O factor 10c [27].

The Fisher immunotype 5 O-polysaccharide has structure 45 [43, 59], and, hence, on this basis it should be included into subgroup O10a,10b and not into O10a,10c as in [1]. The serological status of Fisher 5 still awaits elucidation; although the strain reacts in serum O10c, it may contain non-defined antigens absent from reference strain O10a,10c but present in O10a,10b.

Serogroup O11

This serogroup is subdivided into subgroups O11a,11b and O11a,11c, but the O-specific polysaccharides of the reference strains of both subgroups have identical structure **47** [60]. They are neutral and consist of trisaccharide repeating units containing the residues of D-glucose, N-acetyl-D-fucosamine, and N-acetyl-L-fucosamine.

47 -3)- α -L-FucNAc-(1-3)- β -D-FucNAc-(1-2)- β -D-Glc-(1-

Subgroups O11a,11b and O11a,11c, strains 170015, 170016, Habs 11, Fisher 2

As the polysaccharides proved to be identical, the subdivision of the O11 serogroup into two subgroups remains unsubstantiated. The common O factor 11a is obviously associated with the polysaccharide of structure **47**, whereas the O factors 11b and 11c seem to be related to other heat-stable antigens, which are absent from the purified lipopolysaccharides. It should be noted that a number of strains were found which reacted in both anti-O11b and anti-O11c sera [61, and Lányi, unpublished observation]. Whether or not such cultures represent a third subgroup (O11a,11b,11c), is not elucidated at this time; for epidemiological purposes, the establishment of subgroup O11a,11b,11c does not appear justified.

The Habs O11 polysaccharide also has structure **47**, and the same polysaccharide is characteristic of Fisher 2 [43, 59], in agreement with the serological data [1].

Serogroup O12

The O12 serogroup is not subdivided into subgroups. The O-specific polysaccharide of the reference strain has structure **48** [62]. The trisaccharide repeating units of this polysaccharide involve the residues of N-acetyl-D-quinovosamine, 2-acetamidino-2,6-dideoxy-L-galactose (fucosacetamidine), and 5,7-diacetamido-3,5,7,9-tetradeoxy-D-glycero-L-galacto-nonulosonic acid. A simultaneous presence of the carboxyl and acetamidino functions endows this polymer with amphoteric character.

48 -3)- α -D-QuiNAc-(1-8)- α -Non(NAc)₂-(2-3)- α -L-FucN-(1-



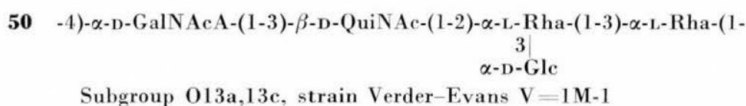
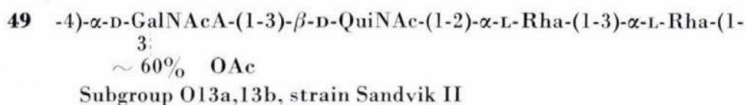
Serogroup O12, strains 170023, Habs 12

The Habs O12 polysaccharide has the same structure in agreement with its serological identity with strain 170023 [1].

Serogroup O13

The O13 serogroup includes two subgroups, O13a,13b and O13a,13c, whose O-specific polysaccharides have structures **49** and **50**, respectively [63]. These polysaccharides possess identical backbone, built up of tetrasaccha-

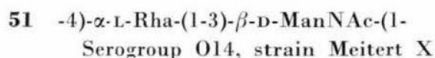
ride repeating units containing two residues of L-rhamnose, a residue of N-acetyl-D-quinovosamine, and a residue of N-acetyl-D-galactosaminuronic acid. In the O13a,13b polysaccharide a part of the residues of N-acetyl-galactosaminuronic acid carries an O-acetyl group at position 3 (degree of O-acetylation cca. 60%), whereas in the O13a,13c polysaccharide O-acetyl groups are absent, but one of the rhamnose residues carries a residue of D-glucose as a side chain. The latter polysaccharide (structure **50**) is the only known branched polymer among *P. aeruginosa* O-specific polysaccharides.



The identity of the backbone of the O-polysaccharides substantiates the combining of these two strains into one serogroup as individual subgroups. The group O factor 13a is evidently associated with a common fragment of the backbone, whereas the O factor 13c seems to be related to the terminal residue of glucose, and the O factor 13b appears to be determined by the O-acetyl group or lack of the glucose residue.

Serogroup O14

The O14 serogroup, which is not subdivided into subgroups, is characterized by the O-specific polysaccharide having structure **51** [64]. It is built up of disaccharide repeating units containing the residues of L-rhamnose and N-acetyl-D-mannosamine.



It is noteworthy that the structure **51** is also characteristic of the backbone of the *Aeromonas salmonicida* O-specific polysaccharide, which involves, in addition, residues of α -maltose as side chains and O-acetyl groups [65].

Serogroup O15

This serogroup, like the preceding one, is not subdivided into subgroups. The corresponding O-specific polysaccharide is also of neutral character and built up of disaccharide repeating units of structure **52**, containing the residues of D-ribose and N-acetyl-D-galactosamine, the former being in the furanose form [66].

52 -2)- β -D-Ribf-(1-4)- α -D-GalNAc-(1-
Serogroup O15, strains 170022, *Serratia marcescens* O14

It is interesting that *Serratia marcescens* O14 and some other representatives of this microorganism produce an O-specific polysaccharide corresponding in structure to 52 [67]. However, no data are available on the serological relationship between *P. aeruginosa* O15 and *S. marcescens* O14.

Discussion

The data summarized above show that most *P. aeruginosa* O-specific polysaccharides have a regular structure built up of linear trisaccharides or tetrasaccharides, and less frequently of disaccharide repeating units; there is only one branched polysaccharide (in subgroup O13a,13c) with a pentasaccharide repeating unit. Several polysaccharides exhibit a masked regularity associated with non-stoichiometric O-acetylation, non-stoichiometric amidation of the carboxyl group or partial epimerization at C-5 of uronic acids. The majority of the polysaccharides are acidic, and in some of them the negative charge is compensated partially or completely by amidation of carboxyl function (serogroup O6) or by introduction of basic acetamidino group (serogroups O2 and O12). The latter endows the polysaccharides with amphoteric properties.

The polysaccharides of different O serogroups differ from one another in monosaccharide composition and structure of their repeating units; they have no common oligosaccharide fragments, and this accounts for the absence of serological cross-reactions between the strains belonging to different O serogroups.

Most of the O serogroups (9 out of 13) of the compiled antigenic scheme are complex, i.e. they are subdivided into two or more subgroups. The subgroups within one serogroup are characterized by having identical or similar monosaccharide composition and the repeating units have the same general architecture, i.e. the same sequence of monosaccharide residues as well as the same modes of substitution and anomeric configurations of most of the sugar residues. As a consequence, these polysaccharides have common oligosaccharide fragments, which determine the serological similarity of strains of different subgroups within one serogroup.

Distinction between subgroups of one O serogroup is associated in seven serogroups with the presence or absence and the position of O-acetyl groups. Among other factors are variations of N-acyl substituents (in two serogroups), configuration of one of the glycosidic linkages (in two serogroups) and mode of substitution of one of the monosaccharide residues (in two serogroups). In some cases the distinction between subgroups is associated with a change

of monosaccharide composition of a polysaccharide; thus one monosaccharide may be replaced by another (in serogroups O2 and O4), or an additional monosaccharide may be attached as a side chain (in serogroup O13).

Conclusion

The data on the structures of the O-specific polysaccharides represent the chemical basis for the classification of *P. aeruginosa*. They are valuable for the improvement of the known serological classification and for the identification of different strains of this microorganism. These data are also necessary for the preparation of synthetic or semisynthetic antigens and artificial vaccines, which may be useful for the immunoprophylaxis, diagnostics and therapy of infections due to *P. aeruginosa*.

Comparison of data for the structures of the O polysaccharides and for the specificity of the corresponding O antigens shows good agreement in most cases. Some contradictions that have been revealed in the course of the comparative study on reference strains from various classification schemes may lead to a further extension or alteration of the compiled scheme. Table III summarizes findings for the "problematic" strains. In particular, it seems ad-

Table III
O antigen reference strains of *P. aeruginosa* awaiting further study

Reference strain	Serological classification	Code for O-polysaccharide structure (see text)	Remarks
Fisher 7	2a,2d	26	In O-polysaccharide structure differs from other subgroups; may have a new subgroup antigen
Wokatsch 14	3a,3b	27	Discrepancy between serological classification and O-polysaccharide structure
170001	3a,3b,3c	27	
Habs 3	3a,3b,3c	28	
Habs 6	6a, . . .	32	Both strains may have partial antigens different from each other and from those of other subgroups
Fisher 1	6a, . . .	37	
Habs 7	7a,7b,7c	—	Absence of O-specific polysaccharide
170020	9a,9b,9d	42	Absence of O-specific polysaccharide in Wokatsch 16; nature of antigens O9c and O9d not clarified
Wokatsch 16	9a,9c	—	
170019	9a,9d	43	
Fisher 5	10a,10c	45	Structure 45 is specific for subgroup O10a,10c
170016	11a,11c	47	O-polysaccharide structures identical in subgroups O11a,11b and O11a,11c; nature of antigens O11b and O11c not clarified

visible to examine Fisher immunotypes 7 and 1 for the presence of prominent new partial antigens that would allow classifying them into separate subgroups of serogroup O2 and O6, respectively. Investigation into the nature of antigens O9c, O9d and O11c may also be of value.

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CYTOTOXIC ACTIVITY OF LYMPHOCYTE SUBPOPULATIONS AGAINST AUTOLOGOUS TUMOUR CELLS IN PATIENTS WITH MYELOID LEUKAEMIAS AND PRELEUKAEMIC DISORDERS

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Autologous lymphocyte populations from different phases of chronic granulocytic leukaemia (CGL), acute myeloid leukaemia (AML) and preleukaemic disorders were compared for cytotoxic activity. ^{51}Cr -release tests showed that T lymphocytes from the quiescent phase of CGL and from the remission phase of AML exerted cytotoxic activity against autologous tumour cells. Such activity was also found in patients with potentially preleukaemic haematological disorders characterized by cytopenia, but not in polycythaemia vera patients. In the majority of cases cytotoxic activity of T lymphocytes could be blocked by native gp70 antigens of gibbon ape leukaemia virus (GaLV) and baboon endogenous virus (BaEV). Blocking effect of carbohydrate-free gp70 as well as p15(E) antigens could be observed less frequently. The role of cell-mediated immune response to oncovirus antigens in the course of myeloproliferative diseases is discussed.

Three different families of human DNA sequences have been described which are related to replication-competent retroviruses isolated from other mammals [1–3]. In addition, retroviral structures including reverse transcriptase and proteins, that cross-react with primate retrovirus structural proteins, have been detected in human leukaemic cells and sera of patients [4, 5]. Endogenous retroviruses can recombine with or promote transcription of certain cellular transforming genes, which may lead to development of malignant tumours [6, 7]. Immune response to retroviral antigens expressed on the surface of tumour cells is suspected of being an important defense mechanism and may be affected through humoral and cellular mechanisms [8]. In a previous report we have demonstrated gibbon ape leukaemia virus (GaLV)- and baboon endogenous virus (BaEV)-specific cytotoxic activity of lymphocytes and blood plasma samples against autologous target cells in patients with myeloid leukaemia and preleukaemia [9]. On the basis of the above findings the aim of the recent study was (i) to identify the lymphocyte

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Table I
Cytotoxic activity of autologous lymphocyte

Cytotoxic activity of lymphocytes							
T-cell							
Patient	% cpm	% residual cpm after treatment with antigens of					
		GaLV			BaEV		
		gp70		p15 (E)	gp70		p15 (E)
		N	G		N	G	
1	70	14	20	70	17	42	70
2	67	17	67	67	20	45	53
3	50	28	50	50	29	38	43
4	48	34	34	48	36	48	48
5	47	27	47	47	31	47	47
6	31	19	31	31	22	31	31

N = native gp70 antigen

G = glycosidase treated gp70 antigen

populations reacting with the primate oncovirus antigens in patients with chronic granulocytic leukaemia (CGL) and acute myeloid leukaemia (AML) as well as potentially preleukaemic haematological disorders; (ii) to investigate the specificity of these reactions and (iii) to demonstrate a possible correlation between oncovirus-specific cellular cytotoxic activities and the course of the disease. For this purpose we have compared the cytotoxic activity of lymphocyte populations in different phases of CGL and AML.

Materials and methods

Separation of cells from human blood. Heparinized blood samples were collected from patients and healthy controls by venipuncture. For removal of the adherent population of mononuclear cells the samples were incubated with carbonyl iron (10mg/ml) for 30 min at 37 °C on a rotary mixer. After incubation the blood was passed twice over a magnet.

Isolation of lymphocytes and myeloid elements was carried out in Ficoll-Uromiro medium (Flow Laboratories Ltd., Irvine, UK) as described by Boyum [10]. Blood plasma, lymphocytes and myeloid elements were separately aspirated. The content of samples taken from each fraction was checked after May-Grünwald-Giemsa staining. The purity of fractions proved to be 95–100% as revealed by haematological investigation.

Isolation of T-cell from the lymphocyte fraction was carried out by method of Bach [11]. In brief, lymphocyte suspension was incubated overnight (+4 °C) with sheep erythrocytes and rosette-forming T cells were separated by repeated Ficoll-Uromiro centrifugation. Non-rosette-forming cells formed a grey-coloured layer at the interface of the dilution buffer and the separating medium. These cells were separately aspirated, too, and further characterized by detection of B-cell markers using FITC-labelled anti-human IgM (Hyland, Costa Mesa, CA, USA) [12], and rosette formation with mouse erythrocytes [13]. Non-rosette-forming lymphocytes were positive in 70–80% for B markers.

Cryopreservation procedure. A suspension of separated lymphocytes was prepared by adding the cells to RPMI 1640 medium (Gibco Bio-Cult Ltd., Paisley, UK) supplemented with 20% heat inactivated fetal calf serum (Gibco Bio-Cult Ltd., Paisley, UK). The portion to be cryopreserved was placed at a concentration of $5-10 \times 10^6$ cells/ml in 10% DMSO on ice in individual 2 ml glass tubes. Then the following cooling protocol was utilized: (i) starting temperature 2 °C to 4 °C; (ii) cooling rate 1 °C/min to -30 °C; (iii) transfer to the liquid nitrogen storage container. When leukaemic disease of a patient had developed into progressive phase, cryopreserved lymphocytes were thawed for cytotoxic assay. After a rapid thawing (37 °C water bath without agitation until all ice crystals melted) the samples were placed at room

subpopulations in the quiescent phase of CGL

Patient	Cytotoxic activity of lymphocytes						
	non-rosette-forming cell						
	% residual cpm after treatment with antigens						
	% cpm	GaLV			BaEV		
		gp70		p15 (E)	gp70		p15 (E)
		N	G		N	G	
1	15	15	15	15	15	15	15
2	0	—	—	—	—	—	—
3	45	31	31	45	45	45	45
4	20	11	20	20	20	20	20
5	0	—	—	—	—	—	—
6	0	—	—	—	—	—	—

temperature for further manipulations. The cells were washed twice with 50 ml RPMI 1640 medium containing 20% FCS. The viable cell count was then determined and the suspension adjusted to the appropriate concentration for cytotoxic test. The trypan blue viability of the cooled and thawed cell samples was $80 \pm 10\%$.

Cytotoxicity test. For study of cell-mediated cytotoxicity the ^{51}Cr -release method was used, as previously described [9]. Viability of target cells and lymphocytes was controlled by trypan blue dye exclusion, and they were used in further experiments only in the case above 90%. For investigation of cell-mediated cytotoxicity 5×10^3 lymphocytes and 5×10^3 ^{51}Cr -labelled autologous target cells were incubated for 4 h. The counts per minute of ^{51}Cr -released into the supernatant were measured in gamma scintillation counter. The per cent cytotoxicity for each sample was calculated as described by Oren et al. [14]. The background control was the percentage of cytotoxicity in tubes containing only target cells.

Purification of gp70 and p15(E) antigens. The baboon endogenous virus propagated on A204 cells (Lot. No. 990 A) was from the Frederick Cancer Research Center (Frederick, MD, USA) and the gibbon ape leukaemia virus propagated in NC37 cells (Lot. No. 18-76) was from the Pfizer Laboratory (Maywood, NJ, USA). Both viruses were provided in purified form through the Office of Resources and Logistics, National Cancer Institute, Bethesda, MD, USA.

A method for isolating pure viral envelopes from C-type RNA viruses [15] was combined with methods for preparation of envelope glycoproteins from Rauscher virus [16, 17] and from endogenous primate retroviruses [18]. Molecular weight of gp70 and p15(E) was determined by SDS-PAGE [19]. Gels were stained for protein with Coomassie brilliant blue and for carbohydrate by the PAS procedure. Protein quantitation was according to Lowry et al. [20].

Glycosidase treatment of viral gp70-s. The procedure described by Ohno et al. [21] was used. Forty μg of a glycosidase mixture (Miles, Frankfurt, FRG) were added to 400 μg of BaEV or GaLV gp70 in 50 mM sodium citrate buffer, pH 4.0. The efficacy of glycosidase treatment was controlled by SDS-PAGE: the glycosidase treated antigens proved to be free of carbohydrate.

Inhibition of cytotoxic reactions by oncoviral envelope antigens. Before adding to target cells, lymphocyte samples were incubated with 20 ng of appropriate native and glycosidase treated gp70 as well as p15(E) antigens at 37 °C for 30 min. Blocking activity of BaEV and GaLV antigens are reflected by the residual cpm after treatment with the appropriate derivatives of oncoviral envelopes.

Results

Table I summarizes the results of cytotoxic studies on 6 patients being in the quiescent phase of CGL. Cytotoxicity of T-cells could be observed more frequently and was of higher degree as compared to that of the non-rosette

Table II
Cytotoxic activity of lymphocyte subpopulations from different

Patient	Source of lymphocytes	Cytotoxic activity of lymphocytes						
		T-cell						
		% cpm	% residual cpm after treatment with antigens of					
			GaLV			BaEV		
			gp70		p15 (E)	gp70		p15 (E)
			N	G		N	G	
1	quiescent phase	75	15	30	75	17	40	75
2		70	20	70	70	30	45	50
3		66	30	66	66	30	40	43
4		52	40	40	52	42	52	52
5		48	27	48	48	28	48	48
6		48	20	48	48	20	48	48
1	blastic crisis	15	15	15	15	15	15	15
2		0	—	—	—	—	—	—
3		0	—	—	—	—	—	—
4		0	—	—	—	—	—	—
5		0	—	—	—	—	—	—
6		0	—	—	—	—	—	—

Abbreviations: see in Table I

forming lymphocytes. The native gp70 antigen of GaLV and BaEV inhibited the T-cell mediated cytotoxic activity in each case. Digestion by glycosidases of the carbohydrate part of gp70 antigens reduced or in some cases abrogated their blocking activity. Inhibitory effect of p15(E) antigens was observed less frequently than that of the native or glycosidase-treated gp70 antigens.

Results of cytotoxic investigation of the some patients suffering from the blastic crisis of CGL are shown in Table II. ⁵¹Cr-labelled myeloblasts collected from the blastic phase of CGL served as target cells. A remarkable difference was observed between the cytotoxic effect of autologous lymphocytes from the blastic crisis and that of cryopreserved lymphocytes from the quiescent phase. Cytotoxicity of lymphocytes from the blastic crisis could be found only in one case. T-cells exerted cytotoxic activity more frequently than non-rosette forming lymphocytes. Blocking pattern by GaLV and BaEV antigens was quite similar to and in some cases almost identical with the quiescent phase studies.

Table III shows the results on 6 patients with AML. Myeloblasts from the progressive phase were used as target cells. A difference could be observed in the cytotoxic activity of lymphocytes from the progressive phase and from remission. T-cells from the progressive phase were inactive. In contrast, cytotoxic activity of the remission phase T lymphocytes was found in each case. Cytotoxic activity of non-rosette-forming cells could be detected only in

phases of CGL against autologous tumour cells of blastic crisis

Patient	Source of lymphocytes	Cytotoxic activity of lymphocytes						
		non-rosette-forming cell						
		% cpm	% residual cpm after treatment with antigens of					
			GaLV			BaEV		
			gp70		p15 (E)	gp70		p15 (E)
			N	G		N	G	
1	quiescent phase	18	18	18	18	18	18	18
2		0	—	—	—	—	—	—
3		30	20	20	30	30	30	30
4		30	15	30	30	30	30	30
5		0	—	—	—	—	—	—
6		0	—	—	—	—	—	—
1	blastic crisis	0	—	—	—	—	—	—
2		0	—	—	—	—	—	—
3		0	—	—	—	—	—	—
4		0	—	—	—	—	—	—
5		0	—	—	—	—	—	—
6		0	—	—	—	—	—	—

one patient, being in the remission phase of the disease. Blocking experiments with BaEV and GaLV antigens gave results resembling CGL studies. The inhibitory effect of glycosidase-treated gp70 as well as p15(E) antigens of BaEV was observed less frequently as compared to GaLV antigens.

Results of studies on 10 potentially preleukaemic patients are demonstrated in Table IV. The preleukaemic disorders were characterized by cytopenia in 6 patients, whereas 4 patients suffered from polycythaemia vera. Autologous cytotoxic activity was demonstrated only in patients with cytopenia. This activity was mediated mainly by the T-cells. The T-cell-dependent immune response could be inhibited more frequently by native and glycosidase-treated gp70 antigens than cytotoxic activity mediated by the non-rosette forming cells.

Cytotoxic activity of lymphocytes against autologous granulocytes could not be detected in 20 healthy control persons. In control experiments cryopreservation procedure of lymphocytes did not influence the degree of their cytotoxic activity.

Discussion

We have found that cytotoxic activity of lymphocytes could be observed frequently in the quiescent phase of CGL, whereas such activity of lymphocytes from the blastic crisis of CGL was found only in one case. Similar relation-

Table III

Cytotoxic activity of lymphocyte populations from different

Patient	Source of lymphocytes	Cytotoxic activity of lymphocytes						
		T-cell						
		% cpm	% residual cpm after treatment with antigens of					
			GaLV			BaEV		
			gp70		p15 (E)	gp70		p15 (E)
			N	G		N	G	
1	blastosis	20	20	20	20	20	20	20
2		0	—	—	—	—	—	—
3		0	—	—	—	—	—	—
4		0	—	—	—	—	—	—
5		0	—	—	—	—	—	—
6		0	—	—	—	—	—	—
1	remission	65	15	25	50	30	30	65
2		50	28	20	50	35	50	50
3		42	21	21	27	25	27	28
4		25	11	20	15	10	20	25
5		20	15	20	20	20	20	20
6		20	10	20	20	20	20	20

Abbreviations: see in Table I

Table IV

Cytotoxic activity of autologous lymphocyte populations in patients

Patient	Diagnosis	Cytotoxic activity of lymphocytes						
		T-cell						
		% cpm	% residual cpm after treatment with antigens of					
			GaLV			BaEV		
			gp70		p15 (E)	gp70		p15 (E)
			N	G		N	G	
1	IRSA	65	3	12	47	5	55	51
2	IRSA	52	8	30	32	44	52	52
3	IRSA	40	11	30	25	5	33	35
4	pancytopenia	32	18	27	32	27	29	32
5	pancytopenia	30	8	17	30	16	21	30
6	pancytopenia	30	25	30	30	30	30	30
7	p.v.	0	—	—	—	—	—	—
8	p.v.	0	—	—	—	—	—	—
9	p.v.	0	—	—	—	—	—	—
10	p.v.	0	—	—	—	—	—	—

N = native gp70 antigen

G = glycosidase treated gp70 antigen

p.v. = polycythaemia vera

IRSA = idiopathic refractory sideroblastic anaemia

phases of AML against autologous myeloblasts

Patient	Source of lymphocytes	Cytotoxic activity of lymphocytes						
		non-rosette-forming cell						
		% residual cpm after treatment with antigens of						
		% cpm	GaLV			BaEV		
			gp70		p15 (E)	gp70		p15 (E)
			N	G		N	G	
1	blastosis	0	—	—	—	—	—	—
2		0	—	—	—	—	—	—
3		0	—	—	—	—	—	—
4		0	—	—	—	—	—	—
5		0	—	—	—	—	—	—
6		0	—	—	—	—	—	—
1	remission	0	—	—	—	—	—	—
2		0	—	—	—	—	—	—
3		35	25	25	28	20	25	34
4		0	—	—	—	—	—	—
5		0	—	—	—	—	—	—
6		0	—	—	—	—	—	—

with potentially preleukaemic haematological disorders

Patient	Diagnosis	Cytotoxic activity of lymphocytes						
		non-rosette-forming cell						
		% residual cpm after treatment with antigens of						
		% cpm	GaLV			BaEV		
			gp70		p15 (E)	gp70		p15 (E)
			N	G		N	G	
1	IRSA	31	19	20	24	31	31	31
2	IRSA	20	20	20	20	20	20	20
3	IRSA	45	13	37	45	45	45	45
4	pancytopenia	32	—	—	—	—	—	—
5	pancytopenia	32	—	—	—	—	—	—
6	pancytopenia	32	—	—	—	—	—	—
7	p.v.	0	—	—	—	—	—	—
8	p.v.	0	—	—	—	—	—	—
9	p.v.	0	—	—	—	—	—	—
10	p.v.	0	—	—	—	—	—	—

ship was observed between the remission and active phase of AML. In addition, data indicated that this immune response was mediated mainly by the T lymphocytes. It has been reported that peripheral blood mononuclear cells, which contain natural killer (NK) population, too, lyse various kinds of cultured tumour cells. However, they hardly lyse freshly isolated tumour cells, particularly those of autologous tumours [22]. Effector cell fraction in our experiments might contain NK lymphocytes, too. This may be a possible explanation of autologous cytotoxic activities without inhibitory effect of GaLV and BaEV antigens. Furthermore, recent results of experiments in our laboratory show that cytotoxic activity of K cells against antibody-coated target cells also might explain the lysis of autologous target cells in some patients with CGL, AML and preleukaemia (unpublished observation). Thus, the presence of antibody-dependent cellular cytotoxicity [23] cannot be excluded. In any case, the sensitized T lymphocytes seem to play primary role in antitumour immune response directed to oncoviral antigens in patients with CGL, AML and preleukaemia.

Which antigens may have involved in generation of such cytotoxic responses? The cytotoxic activity of T-cells could be blocked by native GaLV and BaEV gp70 antigens in the majority of CGL cases. Glycosidase treatment reduced the blocking activity of gp70 antigens, furthermore, immune response to p15(E) antigen could be observed relative rarely. Similar results were obtained in patients with AML, with the exception that glycosidase-treated BaEV gp70 as well as p15(E) antigen exerted blocking effect more rarely, as compared to those of GaLV. It has been reported that virus-induced tumour-specific surface antigens [24] as well as onco-developmental carbohydrate antigens [25] may be also targets of cytotoxic immune response. It is also known that envelope glycoprotein antigens of primate oncoviruses have at least three antigenic determinants [26]. In the light of above findings is reasonable to assume that the residual blocking activity of gp70 envelope antigens after glycosidase treatment as well as p15(E)-specific immune response of lymphocytes indicate the virus-specific nature of the cellular cytotoxicity.

Concerning the third question raised in the introduction, we have observed a remarkable difference between autologous cytotoxic activity exerted by lymphocytes in different phases of CGL and AML. In addition, cytotoxicity of lymphocytes was observed in patients with preleukaemia characterized by cytopenia in contrast to polycythaemia vera. To explain these changes in cytotoxic activity one can hypothesize that envelope antigens of endogenous oncoviruses are shedding from the surface of leukaemic blast cells. Thus, they may block the effector lymphocytes in active phase of AML. The weak immune response detected in relapsus of AML as well as in blastic crisis of CGL may be the consequence of the immunosuppressive effect of leukaemia viruses, too. Similar phenomena might explain the development of preleukaemia into overt

leukaemic disease. Experiments clarifying these possibilities are going on in our laboratory. Data available from our experiments on oncovirus-specific immune response might serve as a tool for monitoring the course of myeloproliferative diseases.

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PRODUCTION OF TOXIC SHOCK SYNDROME TOXIN-1 (TSST-1) ASSOCIATED WITH *STAPHYLOCOCCUS AUREUS* FROM A PYOGENIC SKIN INFECTION

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Toxic Shock Syndrome Toxin-1 (TSST-1) producing *Staphylococcus aureus* associated with TSS was isolated from an abscess on buttock, from a two-year-old male child suffering from diarrhoea, high fever and shock. The isolate was subclassified into *S. aureus* var. *hominis* of CV type-D, and was lysed by phage 75 of Group-III. It was multidrug resistant, exhibited double ring (DR) CVRT pattern and also produced enterotoxin-C (SE-C). Out of 217 *S. aureus* strains from pyodermas, clinical sources, food poisonings and dairy foods, 139 (64.1%) produced enterotoxin. SE-C as single (33.8%) or in combinations (25.2%) was common enterotoxin. None of these strains produced TSST-1.

“Toxic Shock Syndrome (TSS)” was first described by Todd et al. [1] in 1978 in seven children aged between 8–17 years and they isolated *Staphylococcus aureus* from mucosal (nasopharyngeal, vaginal, tracheal) or sequestered (empyema, abscess) sites, but not from blood. TSS in menstruating women using tampons and having *S. aureus* infection has drawn considerable medical attention in the developed countries in recent years [2]. Many workers have reported TSS in association with a wide range staphylococcal infections like surgical wounds [3], septic abortions, fasciitis, osteomyelitis, empyema, abscess [4] and food poisoning [5]. TSS could result from staphylococcal infections at a wide variety of sites in persons of any age and of either sex [3]. The signs and symptoms observed in this illness include diarrhoea, vomiting, high fever, low blood pressure, erythroderma, oedema of the extremities, renal failure and shock. Peripheral desquamation is noted during recovery [6].

Since early 1984, two extracellular toxins of *S. aureus* were reported to be mainly associated with TSS. (i) Staphylococcal enterotoxin-F isolated

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and characterized by Bergdoll et al. [5, 7, 8], is a simple protein with a molecular weight of $24\,000 \pm 500$ and consists of 188 amino acid residues, also been found in breast milk [9], sera, urine and vaginal washings of patients during acute episodes of TSS [10]. (ii) Pyrogenic exotoxin-C isolated and characterized by Schlievert et al. [11, 12] and found to enhance the host susceptibility to lethal endotoxin shock [13].

At the TSS Symposium, Madison, Wisconsin, May 30–June 1, 1984, a tentatively new nomenclature was proposed, to avoid confusion and controversies over this new syndrome by Bergdoll and Schlievert [14], to all the associated toxins and factors of TSS as "Toxic Shock Syndrome Toxin-1 (TSST-1)". This nomenclature has been used throughout the discussion of this report.

We report a case, clinically and serologically diagnosed as TSS associated with *S. aureus*, isolated from a pyogenic skin infection. This appears to be the first report of *S. aureus* associated TSS and a serological identification of TSST-1 marker, from the Asian countries.

Case history

During July, 1985, a two-year-old boy, after an injection at a private clinic, developed an abscess, at the site of the needle prick, on the right side of his buttock, and was brought to the Osmania General Hospital, Hyderabad. The infection started as a nodule and became purulent with a complaint of severe pain and throbbing sensation. Within three days, there was a sudden onset of watery diarrhoea, high fever, hypotension and mild shock. The patient was kept on bactrim treatment and he recovered. During the phase of recovery there was desquamation on his soles and palms.

Materials and methods

Identification and typing of S. aureus. Standard procedures were followed to identify the isolate [15, 16]. The phage typing of the isolate was carried out at the ICMR Staphylococcal Phage typing centre MAMC, New Delhi.

Subspecies typing. The test was performed as devised by Meyer [17]. *S. aureus* was differentiated based on host origin (human, bovine and canine). The pigmented growth of *S. aureus* on nutrient agar containing crystal violet (Merck, Germany) to a dilution of 1:100 000 was the base for the subspecies typing and conclusive with the support of results of coagulase activity on human and bovine plasma, haemolysin typing and phage typing [17, 18].

Kirby–Bauer's single disc method [19], was employed for detecting drug sensitivity of the isolate to penicillin, ampicillin, cloxacillin, erythromycin, garamycin, bacitracin, cephaloridine, lincomycin, chloramphenicol, tetracycline and bactrim.

Crystal violet ring test (CVRT) [20, 21], was originally devised by Borowski and Jakubiec for the detection of the drug sensitivity patterns of hospital *S. aureus* strains [20]. CVRT modified by Rajyalakshmi and Naidu was employed in the current experiment [22]. The results are read as Double ring (DR) pattern, Single ring (SR) pattern and Without ring (WR) pattern and compared with that of penicillin-G sensitivity of *S. aureus* on the same plate. This finding was correlated with the drug sensitivity.

Production of Toxic Shock Syndrome Toxin-1 (TSST-1) and Staphylococcal Enterotoxins (SE). Dialysis sac culture method of Donnelly et al. [23] was employed. A 40×40 cm dialysis paper was thoroughly washed with double distilled water and a 30 ml of double strength brain heart infusion (BHI) broth was put in and tightly knotted into a sac. This was placed in a 250 ml Erlenmeyer flask and 10 ml of phosphate buffered saline (PBS) (0.02 M; pH 7.4) was introduced outside the sac. This assembly was autoclaved at 121 °C for 15 min. The 24-hour-old test culture was scrapped from blood agar and inoculated in PBS outside the sac

and incubated at 37 °C in a Culture Growth Study Chamber (IEC, Bombay) with a gentle shaking of 50 rpm. After 24 h, the liquid surrounding the sac was pooled into a sterile centrifuge tube and spun at 5000 g for 30 min. The clear supernatant was aspirated into a 25 ml sterile vial, preserved with 1:10 000 parts of thiomersal (BDH) and tested for the presence of TSST-1 and SE.

The production of TSST-1 and SE was detected by optimal sensitivity plate (OSP) technique [24, 25]. The anti-TSST-1 (when supplied in 1984 was designated as anti SE-F) and reference SE and anti-SE were supplied by Professor M. S. Bergdoll. Precipitin lines were read after 18 h incubation at 37 °C in a humidified box.

Alongwith the strain isolated from TSS, a further 217 *S. aureus* strains isolated from various sources (food poisonings, 7; pyodermas, 70; other clinical sources, 52 and dairy foods, 88) were also tested for the production of TSST-1 and SE.

Results

Characterization of TSST-1 producing strain. On blood agar plate large, entire colonies were observed. Microscopic studies revealed the presence of Gram-positive, clustered cocci. The isolate produced coagulase (only against human plasma), thermonuclease, alkaline phosphatase, gelatinase, catalase; fermented mannitol aerobically and anaerobically, was tolerant to 10% NaCl, produced orange yellow pigment on staphylococcus medium 110. Hence, the strain was characterized and identified as *S. aureus*, and was designated as strain No. IPM-98.

According to the subspecies typing the isolate was *S. aureus* var. *hominis*. It produced blue coloured growth on crystal violet agar and was read as CV type-D. The strain was lysed by phage 75 of Group-III. It exhibited double ring (DR) pattern and was found resistant to penicillin-G, to ampicillin, cloxacillin, garamycin and tetracycline; intermediate to bacitracin, erythromycin and chloramphenicol; sensitive to lincomycin, cephaloridine and bactrim.

Production of TSST-1. The *S. aureus* strain (IPM-98) produced TSST-1 (Fig. 1) and SE-C. Based on the clinical and laboratory findings the case was diagnosed as TSS associated with a TSST-1 producing *S. aureus*. Bacterial and viral aetiology of diarrhoea could not be traced in laboratory investigation.

Frequency of TSST-1 and SE production. None of the 217 *S. aureus* strains isolated from diverse sources, produced TSST-1, but produced SE in single and multiple combinations. SE-C producers were found common in all different groups except for the strains associated with food poisoning (Table I).

Discussion

Although TSS was not described until 1978 [1], case reports similar to TSS were reported as far back as 1927 by Stevens [26], as an occurrence of *S. aureus* infection with a scarlatiniform rash. In 1942, Aranow and Wood [27], reported a case of a 15-year-old girl with erythroderma, conjunctival hyperaemia, shock and renal failure and severe peeling of the palms and soles

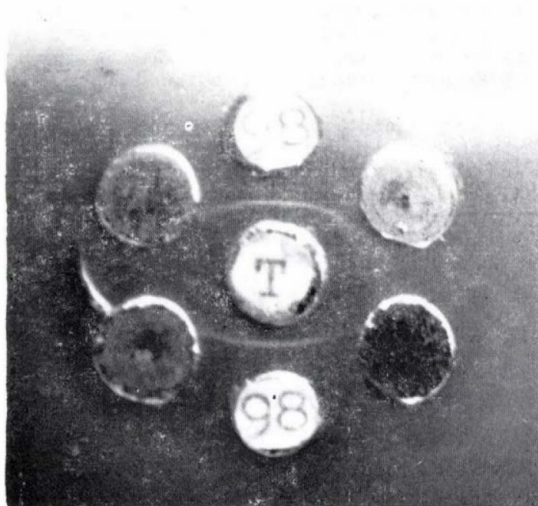


Fig. 1. Detection of TSST-1 by OSP method

Table I

TSST-1 and SE production in S. aureus isolated from diverse sources

Toxin type	Pyodermas	Other clinical sources	Food poisonings	Dairy food
TSST-1	—	—	—	—
SE-A	4 (12.1)	5 (21.7)	5 (71.4)	11 (14.5)
SE-B	4 (12.1)	2 (8.7)	2 (28.6)	7 (9.2)
SE-C	16 (48.5)	12 (52.2)	—	19 (25.0)
SE-D	1 (3.03)	2 (8.7)	—	4 (5.3)
SE-E	—	—	—	1 (1.3)
SE-A + B	—	1 (4.35)	—	1 (1.3)
SE-A + C	6 (18.2)	1 (4.35)	—	9 (11.8)
SE-A + D	—	—	—	2 (2.6)
SE-B + C	1 (3.03)	—	—	5 (6.6)
SE-B + D	—	—	—	3 (4.0)
SE-C + D	1 (3.03)	—	—	6 (7.9)
SE-A + B + C	—	—	—	4 (5.3)
SE-A + B + D	—	—	—	1 (1.3)
SE-A + C + D	—	—	—	1 (1.3)
SE-A + B + C + D	—	—	—	2 (2.6)
SE Producers	33 (47.1)	23 (44.2)	7 (100.0)	76 (86.4)
SE Non-Producers	37 (52.9)	29 (55.8)	—	12 (13.6)
Total strains	70	52	7	88

Overall strains tested for TSST-1 and SE production = 217

Total TSST-1 producers = Nil

Total SE producers = 139 (64.1%)

during convalescence. Recently, Bergdoll et al. [7], demonstrated the production of TSST-1 (SE-F) in a *S. aureus* strain isolated in 1947, indicating that this toxin existed undiscovered since long time.

In this study *S. aureus* (IPM-98 strain) isolated from TSS showed no animal associations and was proved to have originated from a human source. In a recent study at this laboratory zoonosis has been observed to a remarkable extent in pyogenic skin infections. The animal associations and the staphylococcal isolations from pyodermas were found absolutely related (manuscript in preparation).

Non-TSS strains from diverse sources like clinical materials, foods and animals also may produce TSST-1 [7]. In the present study none of the 217 *S. aureus* isolates from diverse sources produced TSST-1. However, 139 (64.1%) out of 217 strains produced SE in single and multiple combinations. SE-C was found, as the most common enterotoxin in single and in combinations in all the different sources, i.e. pyodermas (23, 69.7%); other clinical sources (13, 56.5%); and dairy foods (46, 60.5%); except isolates from food poisoning cases wherein SE-A and SE-B were common. The *S. aureus* (IPM-98 strain) producing TSST-1 also produced SE-C.

In a similar investigation by a US study group [5], a total of 284 strains from patients with staphylococcal illness in hospitals, when examined for the production of TSST-1 and SE, they found that 29 (10.2%) strains produced TSST-1, whereas 128 (45.1%) of the strains produced one or more of SE. Six strains that produced TSST-1 were isolated from foods implicated in food poisoning outbreaks, all but one strain produced SE-A. One strain produced TSST-1 only and development of symptoms were delayed, indicating that the organism may have colonized the intestinal tract and produced enough TSST-1 to cause vomiting and diarrhoea.

The same group also reported the association of SE-C with TSST-1 in 19 cases of TSS (SE-C + TSST-1 in 14 cases; SE-A and SE-C + TSST-1 in 4 cases; SE-C and SE-E + TSST-1 in 1 case).

Workers from various laboratories [6, 28], reported SE-F (TSST-1) as a marker for TSS associated with *S. aureus*, whereas the serological absence of anti SE-F (Anti TSST-1) has been shown to be a marker for susceptibility of persons to TSS. The populations of developing countries have higher antibody titres to at least one enterotoxin SE-B than do persons from developed countries [29]. German population contained relatively high antibody titre to SE-F (TSST-1) [5].

Since staphylococcal infections are quite common in developing countries it may be speculated that this population has higher antibody titre to TSST-1 than the people in the developed countries [6]. This may be the reason that TSS has so far not been reported from Asian or South American or from any of the developing countries.

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IMPRINTING BY NON-SIGNAL PROTEIN MOLECULE IN *TETRAHYMENA*

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Bovine serum albumin (BSA) enhances the division of *Tetrahymena* and leaves negative imprinting. In consequence, a repeated meeting with BSA 1, 2 or 3 weeks after the first one is followed by a significantly reduced division rate of *Tetrahymena* cells. The imprinting is fairly specific for BSA; the division rate was not influenced significantly by either human serum albumin or an anti-rabbit sheep immunoglobulin.

The unicellular organism *Tetrahymena*, living in an ever varying environment, must respond to a great number of substances. To give the correct response, it must memorize the usefulness or harmfulness of the substances ever met with [1, 2]. If it meets a signal molecule, e.g. a hormone or hormone-like substance of higher organisms, the signal molecule leaves a hormonal imprinting behind, similar to that coming about in the higher organism in the perinatal period. The effect of imprinting, a changed reactivity of the unicellular, manifests itself through many, even 500, generations [3]. This means that *Tetrahymena* memorizes the event of its first meeting with a signal molecule [4]. The changes in its reactivity can be estimated by measuring the changes in its specific hormone-binding capacity and those in the response given to the signal molecule [5].

We have shown experimentally [6] that molecules being not signal molecules in higher organisms but having a characteristic stereoconfiguration may also be capable of imprinting in *Tetrahymena*. The imprinting by such molecules is less pronounced and lasts shorter than hormonal imprinting.

In the present studies, we wished to see to what a degree *Tetrahymena* is capable of memorizing the first meeting with a general protein, bovine serum albumin (BSA), and how specific this memorization is if exists at all.

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Materials and methods

Cultivation. The GL strain of *Tetrahymena pyriformis* was maintained at 27 °C in a medium containing 0.1% yeast extract and 1% Bacto Tryptone. Mass cultures were pretreated with 5 µg/ml BSA for 24 h. The pretreated cultures were transferred to BSA-free medium and tested for reactivity with BSA (Phylaxia, Budapest), human serum albumin (HSA, Serva, Heidelberg) and anti-rabbit sheep immunoglobulin (IgG, Human Budapest) at one week intervals (1, 2 and 3 weeks after BSA treatment). Changed reactivity, i.e. changed cell division rate, was used as indicator of imprinting.

A series of experiments was performed. In each experiment *Tetrahymena* were treated with only a part of the treatment combinations.

Statistical analysis. Taking the great variability between experiments into consideration, we determined (i) the differences of the means related to the treatment combinations and (ii) the standard deviations for the differences separately for each experiment, using analysis of variance. The overall mean difference for a given treatment-combination pair, weighting by invariance, the $p = 0.95$ confidence limits and the significance of the deviations were determined for all the possible treatment-combination pairs. Taking into account the great number of possible combinations, we choose $p = 0.01$ as limit of significance.

Cell counting. Cells were enumerated in seven capillaries in each experiment. The experiments were repeated at least five times, at most 25 times. Thus, each column in Fig. 1 represents the mean for at least 35 measurements; the results are related to the absolute control as unit.

Results and discussion

Imprinting with signal molecules usually induces an upward deviation of both the binding index for the signal molecule and the functional indices, i.e., both the binding to the unicellular of the signal molecule and the functional capacity of the latter are enhanced. The same was found [6] when binding values for BSA as imprinter were examined. In the present study the multiplication of the *Tetrahymena* cells was enhanced by the first meeting indeed, however, it was reduced by a repeated meeting. This means that BSA left a negative imprinting behind.

A single meeting with BSA (Fig. 1) was followed by a long-lasting effect, viz., the cell-division rate was still at a significantly reduced level one week after the treatment (BSA/C, vs. C/BSA). Moreover, a second meeting with the same substance was followed by a significantly reduced division rate even if the second treatment was performed as late as three weeks after the

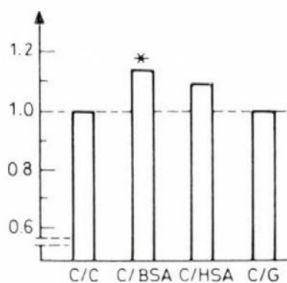


Fig. 1. Effect of BSA, HSA and IgG treatments on *Tetrahymena* cultures. The average cell count for the absolute control (C/C) is taken as unit. C = no treatments, BSA = treatment with bovine serum albumin, HSA = treatment with human serum albumin, G = treatment with anti-rabbit globulin. ($P < 0.01$ related to C/C)

first meeting (Fig. 2). It would not be justified to speak of an oblivion, for the lowest multiplication rate was measured on the occasion of the second testing, i.e. two weeks, not one week, after the first meeting. There is no doubt that the pretreated *Tetrahymena* memorized the experience of the first meeting and passed the experience to its progeny. It should be noted that *Tetrahymena*

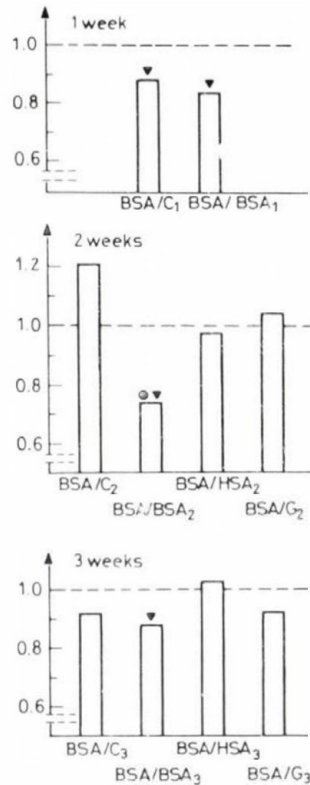


Fig. 2. Effect of BSA, HSA and IgG treatments on *Tetrahymena* cultures pretreated with BSA 1, 2 or 3 weeks before. The average cell count for the absolute control (C/C) is taken as unit. Numerator indicates the first, denominator indicates the second treatment. C = no treatments, BSA = treatment with bovine serum albumin, HSA = treatment with human serum albumin, G = treatment with anti-rabbit globulin. The index to the denominator indicates the interval in weeks between first treatment and second treatment or testing without repeated treatment. ($p < 0.01$ + related to C/C, related to C/BSA see in Fig. 1)

cells divide five to six times daily, that is, the 100th to 120th generations were examined after three weeks of incubation.

As we have already seen, the cell division rate was still reduced one week after a single BSA treatment (Fig. 2). One might assume that in this case it was not the single meeting that reduced the division rate (for there was no significant difference between the value of BSA/C₁ and that of BSA/BSA₁). Two or three weeks after the single meeting (BSA/C₂ and BSA/C₃), however,

the effect on cell multiplication had already ceased, nevertheless, the second BSA treatment at two weeks (BSA/BSA₂) was followed by the greatest reduction of the multiplication.

There is no doubt that the unicellular organism *Tetrahymena* recognizes, and reminds of, proteins that represent no signal in the higher organism. This is not surprising because it is not sure that even a molecule of hormone nature in higher animals has the same meaning for an unicellular. Possibly, they signal only peptide molecules. It is also possible, on the other hand, that the direction of imprinting has some meaning, for, as mentioned above, the first meeting with a hormone is followed by a positive imprinting.

We performed experiments with HSA and IgG to show whether *Tetrahymena* memorizes "protein in general" or a protein of given stereoconfiguration and amino-acid sequence, in this case: BSA. Figure 2 shows that HSA tended to increase the cell division rate (BSA/HSA₃ vs. BSA/C₃), however, the increment was not significant. The challenge with globulin was fully indifferent (BSA/G₃ vs. BSA/C₃). The fact that the proteins of different structure given two or three weeks after BSA treatment provoked no significant change indicates that the protein molecule once met by the unicellular is distinguished from other proteins by the latter.

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ENHANCEMENT OF CILIA REGENERATION BY HORMONE TREATMENT OF *TETRAHYMENA*

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Regeneration of cilia of deciliated *Tetrahymena* was modified by hormones added to the nutrient medium; it was enhanced by serotonin (an amino-acid type substance), insulin (a polypeptide-type hormone) and prednisolone (a steroid-type hormone). Diiodotyrosine, another amino-acid type hormone, did not influence regeneration. Prednisolone, a hormone morphogenetic in mammals appeared to be the most active in regenerating cilia.

In the cell membrane of *Tetrahymena*, there are patterns that bind to hormones of higher animals, and hormone-binding loci are formed on the effect of hormones [1]. The hormone-binding structures behave like receptors, mediating the hormone-carried information intracellularly [2]. The phagocytotic capacity of the unicellular [3], like that of cells of higher organisms [4–6], is increased by histamine and serotonin, and the unicellular makes distinction between these amines and their analogues (precursors) [7]. The cell division of *Tetrahymena* is stimulated by diiodotyrosine (DIT) as well as by serotonin [8], while the sugar uptake of the unicellular is increased by adrenaline and insulin [9]. Its RNA synthesis is influenced by certain hormones of polypeptide character [10], whereas among the steroid hormones deoxycorticosterone and prednisolone have been found to be not indifferent from the aspect of the phagocytosis of the unicellular [11]. There is not doubt that the life processes in whole *Tetrahymena* are different from those of higher organisms, nevertheless, its biochemical processes, similarly to those of the latter, can be influenced by hormones.

The cilia represent the organ of locomotion of *Tetrahymena*; they play a basic role in the life activity of the unicellular. Removed cilia will regenerate [12–15]. Regeneration of cilia is a morphogenetic process which can be influenced by hormones in higher organisms. In the present studies, the effects of four hormones on the regeneration of cilia were investigated in *Tetrahymena*.

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Materials and methods

Tetrahymena. The GL strain of *Tetrahymena pyriformis* was maintained under continuous shaking in a water bath of 28 °C. The medium contained 2% Bacto Tryptone (Oxoid, England) and 0.2% yeast extract. Two-day cultures, i.e. cultures in the logarithmic phase, were used in the experiments.

Regeneration of cilia. *Tetrahymena* were deciliated as described by Rosenbaum and Carlson [16]. The deciliated organisms were cultured under continuous shaking in a water bath of 28 °C. The nutrient medium amounted to 20-fold the organism's volume. The regeneration of cilia, in the presence and absence of hormone, was examined after 20, 40, 60, 80, 100, 120 and 150 min of incubation.

Hormones. The following hormones were used: prednisolone (10^{-8} M; Di-Adreson F aquosum, Organon, the Netherlands), insulin (10^{-6} M; Semilente MC Novo, Copenhagen, Denmark), serotonin (10^{-8} M, serotonin creatinine sulphate, Reanal, Hungary), diiodotyrosine (DIT, Fluka, Switzerland).

Motility of cells. Samples taken at intervals were examined for motility of *Tetrahymena*,

$$\text{Motility per cent} = 100 \frac{\text{no. of moving cells}}{\text{no. of cells observed}}$$

Moving and immobile cells were enumerated in a Fuchs-Rosenthal chamber. The immobile living cells lacking cilia were distinguished from dead cells by the trypan blue method.

Statistical analysis. Each experiment was repeated five times. In the first step, the data belonging to the same control were comprised within the same group. In the second step, the frequencies obtained for the treated group were compared to those simultaneously obtained in the control group; the Mantel-Haenszel test was used. Two global hypotheses were tested: (i) whether the frequencies in the same group were different from the controls at all; (ii) whether the deviations obtained at different times were of the same direction as measured by the Mantel-Haenszel test.

Results and discussion

The regeneration of cilia as expressed in motility per cent is shown in Fig. 1. The regeneration rate was increased by each of the hormones except DIT. The insulin curve seems to be the steepest; however, due to a high standard deviation, the difference is less significant for insulin than for prednisolone. Of the single motility per cent values of the prednisolone curve three, while of those of each of the insulin and serotonin curves only one proved to be significantly different from the respective control value.

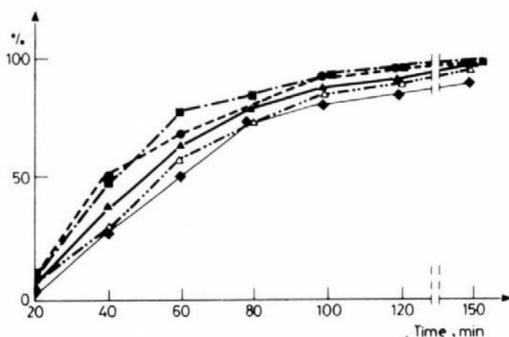


Fig. 1. Effect of hormones on the regeneration of *Tetrahymena* cilia. Ordinate, motility per cent; abscissa, regeneration time. $\triangle$ — $\triangle$ control; $\diamond$ — $\diamond$ DIT; $\blacktriangle$ — $\blacktriangle$ prednisolone; $\bullet$ — $\bullet$ serotonin; $\blacksquare$ — $\blacksquare$ insulin

The global values for each of prednisolone ($p < 0.001$), insulin ($p < 0.01$) and serotonin ($p < 0.05$) proved to be significantly different from the control value.

The above four hormones were chosen to represent amino-acid-type, polypeptide-type and steroid hormone(s). Although some morphogenetic effect was achieved with each type of hormones in *Tetrahymena*, it cannot be attributed to chance alone that prednisolone, a hormone belonging to the morphogenetic hormones of higher animals, exerted the most significant effect in *Tetrahymena* [17].

In higher animals, insulin and serotonin act on membrane receptors, while prednisolone, a glucocorticoid, has a cytosol receptor. In an earlier study, we detected insulin binding to membrane receptor in *Tetrahymena* [18], however, this organism has no receptor of cytosol type, except if it has been treated with steroid hormone for a long time [19]. In our present experiments, hormones were not present for long times, therefore, another mechanism must be assumed. It may be proposed that the cilia regeneration was provoked by binding to some membrane receptor (similar mechanism has already been reported [20, 21] or by a mechanism acting at a level other than receptor.

In the case of polypeptide- and amino-acid-type hormones, one might imagine that *Tetrahymena* used these hormones as food and the enhanced regeneration of cilia might be attributed to an improved nutrition. However, taking into account the low level of the hormones applied in the experiments and the fact that the nutrient medium was abundant in nutrients speaks against this assumption.

DIT, a member of the tyrosine series, the precursor of thyroxine, is another hormone of metamorphic effect. It has proved to be more efficient than thyroxine as stimulator of the cell division of *Tetrahymena* [8]. It seems that such a "morphogenetic" activity manifests itself in stimulating cell division, but not in the regeneration of cilia.

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BACTERIAL TRANSLOCATION IN DIANHYDRODULCITOL-TREATED MICE

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Escherichia, *Proteus*, *Klebsiella* and *Streptococcus* strains were isolated from mesenteric lymph nodes, spleens and livers of conventional mice treated with dianhydrodulcitol (DAD), indicating that intestinal bacteria had appeared in organs usually containing no bacteria. The frequency of bacterial translocation showed direct relation to the dose of the drug and appeared simultaneously with the spleen atrophy caused by DAD.

In certain pathological conditions, e.g. enhanced permeability of the intestinal mucosa, excess of some bacterial species in the gut or impaired function of the immune system, members of the intestinal flora may permeate the intestinal wall and be translocated into mesenteric lymph nodes and other organs [1].

In our earlier experiments [2], germfree mice proved to be more resistant, and conventional mice more sensitive than SPF ones to the lethal effect of dianhydrodulcitol (DAD). DAD is an alkylating agent, used as a lymphotropic cytostatic. Large doses of this drug impair the intestinal mucosa [2, 3] and increase intestinal permeability. Furthermore, DAD has proved to be immunosuppressive in both large doses and long-term experiments [4, 5]. It is therefore reasonable to assume that the increased sensitivity of conventional mice to DAD may be attributed to bacterial translocation.

Our previous experiments have also shown [2, 5] that DAD-treated mice develop a severe spleen atrophy simultaneously with the immunosuppressive state, i.e. 4–6 days after a single large dose and in the second week of long-term experiments.

In the present study, we searched for intestinal bacteria supposedly translocated during the immunosuppressive state in otherwise bacteriologically negative organs of DAD-treated conventional mice.

Materials and methods

Experimental animals. Two-month-old CFLP (LATI, Gödöllő, Hungary) conventional mice of both sexes were used.

Dianhydrotolucitol treatment. The cytostatic agent (Chinoin Pharmaceutical Works Ltd., Budapest) was dissolved in sterile distilled water not more than 30 min before the experiment. Control mice were given sterile PBS instead of DAD.

Cell suspensions. Two mesenteric lymph nodes, the spleen and the liver were aseptically removed from sacrificed mice. Cell suspension was prepared from the lymph nodes and the spleen as well as from a 100 mg fragment of the liver; each sample was diluted in 0.5 ml PBS.

Isolation of bacteria. The entire amount of each cell suspension was poured into serum broth medium, from which five subcultures were made on blood agar medium at 24 h intervals. The colonies growing in the subcultures were examined macroscopically, microscopically and biochemically. A result was considered negative if no bacterial growth had appeared on any of the five subcultures by the 48th hour of incubation.

Relative spleen weight. The bodies and, after necropsy, the spleens of the mice killed or spontaneously died were weighed.

$$\text{Relative spleen weight} = \frac{\text{spleen weight (mg)}}{\text{body weight (g)}}$$

Spleen index. The spleen index for an experimental group of mice is the quotient of their relative mean spleen weight and relative mean spleen weight for the corresponding control group.

Results

Two series of experiments were performed. In experiment I, DAD was administered in a single dose of approximately LD₅₀ per mouse, i.e. 15 mg DAD per kg body weight, or in a sublethal dose (12 mg/kg). The LD₅₀ had been determined in experiments in which SPF mice were used. In experiment II, 1.5 mg/kg DAD was administered on 11 occasions.

Experiment I. In the first part of this experiment 40 mice were given 15 mg/kg DAD, while another 40, the control group, received the same volume of PBS. Twenty of the DAD-treated mice were examined for mortality. Of these, 9 (45%) died on day 5 or day 6 of observation; the others survived the experiments and were sacrificed on day 14. Ten of the control mice were sacrificed on day 5 to serve in calculating the spleen index for the succumbed mice, another 10, all surviving the observation period were sacrificed on day 14.

From the remaining 20 DAD-treated and 20 control mice isolation of bacteria was attempted. For this purpose, four mice were sacrificed in both groups in each of days 3, 4, 5, 6 and 7 after treatment. The mesenteric lymph nodes, the spleens and the livers were tested. No bacteria could be isolated from the controls. The isolations from the DAD-treated mice are shown in Table I.

It is clearly seen that the isolations from the organs of the mice sacrificed at the time when other mice died, i.e. on days 5–7, were positive in 91 to 100%. Furthermore, a severe spleen atrophy was demonstrated on the 5th and 6th days following DAD treatment. The high spleen index (1.8) obtained on day 14, on the other hand, speaks for spleen hypertrophy (Fig. 1).

Table I*Bacterial translocation in mice given 15 mg/kg DAD in one dose*

Organs	No. of positive organs/examined organs on day			
	4	5	6	7
	after DAD treatment			
Lymph nodes	0/4	4/4	4/4	3/4
Spleen	0/4	4/4	4/4	4/4
Liver	0/4	3/4	4/4	4/4
Totals	0/12	11/12	12/12	11/12
% positive	0	91	100	91

In the second part of experiment I, 130 mice were given 12 mg/kg DAD (a sublethal dose). As control, another 130 mice were injected at the same time with the same volume of PBS. Ten mice from both groups were observed till the end of the experiment, i.e. the 21st day. None of these mice died during the observation period. Further 10 mice treated with DAD and 10 control mice were sacrificed on each of days 1, 5, 10 and 14 after treatment. The relative spleen weights and the spleen indices were determined. Of the remaining 80 treated and 80 control mice, 8 from each group were sacrificed on different days between day 3 and day 14 after treatment. All the control tests were negative. The isolations from the DAD-treated group are shown in Table II

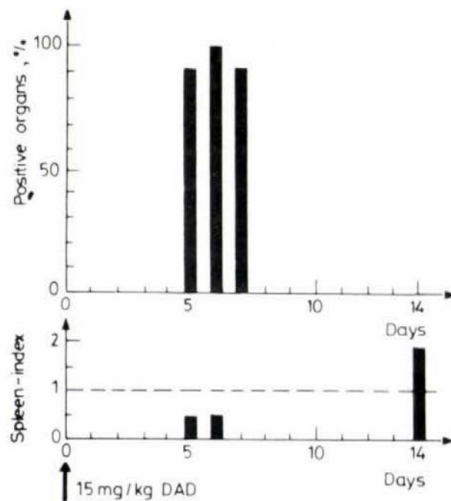


Fig 1. Rates of positive bacteriological tests and spleen indices for mice treated with 15 mg/kg DAD in one dose

Table II
Bacterial translocation in mice given 12 mg/kg DAD in one dose

Organs	Number of positive organs on day										Altogether	
											no. §	%
	3	4	5	6	7	10	11	12	13	14		
	after DAD treatment											
Lymph nodes	4	1	7	6	7	5	4	2	3	2	41	51
Spleen	3	3	6	5	7	6	2	2	2	1	37	46
Liver	1	3	6	6	5	8	2	0	0	0	31	39
Totals	8	7	19	17	19	19	8	4	5	3	109*	—
% positive	33	29	79	70	79	79	33	16	20	12	—	45

□ Eight organs were examined in each group

§ Positive organs/eighty organs

* Positive organs/240 organs

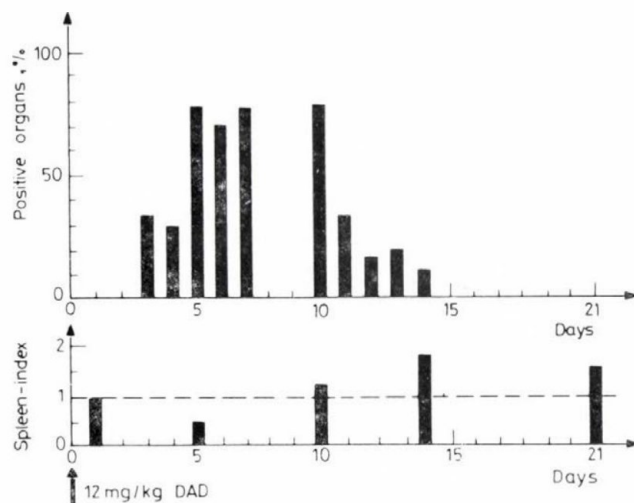


Fig. 2. Rates of positive bacteriological tests and spleen indices for mice treated with 12 mg/kg DAD in one dose

Seventy to 79% of the organs tested gave positive results on days 5–10 following DAD treatment. Lymph nodes yielded the highest isolation rate. The liver samples taken after day 11 were negative.

The percentage positivity and the spleen indices are presented in the function of the time in Fig. 2. The spleen indices for day 5 are suggestive of a severe spleen atrophy, those for day 10 show no considerable difference from the initial spleen index, whereas those for days 14 and 21 are characteristic of a pronounced spleen hypertrophy. The high percentage positivity of the bacteriological cultures coincided with, or immediately followed, the spleen atrophy. As spleen hypertrophy had developed, bacteriologically positive organs began to decrease in number.

From the mice cultured bacteriologically in experiment I, 165 strains were isolated. Of these, 86 (52%) proved to be *Escherichia*, 34 (21%) *Klebsiella*, 35 (21%) *Streptococcus*, 7 (4%) *Proteus* and 3 (2%) other species.

Experiment II. In this experiment, 11 doses of DAD, 1.5 mg/kg dose, were administered to mice on days 1, 4, 5, 6, 7, 8, 11, 12, 13, 14 and 15 of the experiment. Fifty-two mice received DAD and another 52 received PBS. Ten mice of both groups were observed up to day 21. All these animals survived the experiment. Then they were sacrificed. Eight mice were sacrificed on each of days 1, 12 and 15. The relative spleen weights and the spleen index values were calculated for the mice sacrificed on different days.

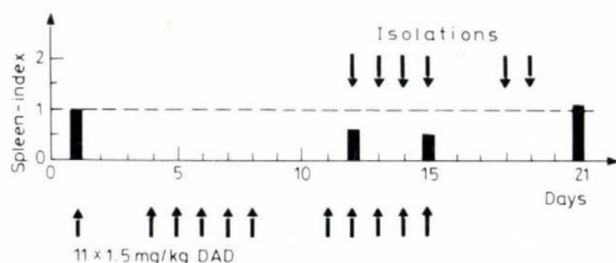


Fig. 3. Long-term experiment using 11 doses of DAD. Spleen indices (columns), the days of isolation of bacteria (upper arrows)

Isolation of bacteria was attempted from mice sacrificed at different times. Three mice were sacrificed on each occasion, viz. on days 12, 13, 14 and 15, i.e. after the 7th DAD injection, and on days 18 and 19, i.e. after the end of treatment. Isolation was attempted from the lymph nodes, the spleens and the livers. Figure 3 presents the spleen indices for the mice sacrificed on the indicated days and the times of bacteriological testings.

Bacteria were isolated from three of the DAD-treated mice (5.5%), in each case from lymph node, in one case from a sample taken on day 14 and in two cases from samples taken on day 15. The spleen and liver samples as well as all the control samples were negative bacteriologically.

The spleen atrophy was the most severe in the mice sacrificed at the end of the treatment, on day 15. In this long-term experiment, too, the positive bacteriological findings coincided with the spleen atrophy. Of the three isolates, two proved to be *E. coli*, the third belonged to the genus *Streptococcus*.

Discussion

Our experiments indicate that translocation of bacteria of the normal intestinal flora into otherwise bacteriologically negative organs occurs on the effect of DAD treatment in conventional mice. This agrees well with a report [6] suggesting that translocation can be demonstrated in SPF animals with

cyclophosphamide, another alkylating agent. As shown by the present results, the frequency of translocation in DAD-treated mice is dose-dependent and the translocation coincides with the spleen atrophy following DAD treatment. According to our earlier experiences [2, 4], DAD dosages approximating the LD₀₅ induce an immunodeficient state accompanied by histologically demonstrable severe inflammation, oedema and mucosal necrosis of the intestinal wall. The translocation following a single large dose of DAD might be explained by the combined effect of intestinal wall damage and immunodeficiency. The spleen hypertrophy following spleen atrophy may be considered to be a response serving the elimination of the heavy bacterial invasion. The latter assumption is supported by our unpublished experience, viz., spleen hypertrophy was not demonstrable in DAD-treated germfree mice.

The translocation was conspicuously less pronounced in the long-term experiment than after administration of a single dose of DAD. Its simultaneous appearance with the spleen atrophy in the long-term experiment can be explained with the immunosuppressive effect of DAD, i.e. the existence of an immunodeficient status in the DAD-treated mice. In the long-term experiment, spleen hypertrophy could not be observed, supposedly because translocation occurred much less frequently than in the short-term experiments.

The bacterial species isolated from the organs belong to the normal intestinal flora of the mouse, they being common residents of the murine colon and caecum.

Our experimental results indicate that, under conventional conditions, the toxic effect of DAD is exaggerated by the bacterial translocation, which, due to the immunosuppression and the intestinal impairment, consistently appears in DAD-treated mice. The relative resistance to DAD of germfree mice, a phenomenon reported by us earlier [2], can be explained by the fact that bacterial translocation is impossible in germfree animals.

The importance of the present results in the human medicine should also be considered, especially with regard to the fact that an environment poor in microbes is favourable in wards where DAD or similar agents are being used as cytostatics. In this respect, it may be stressed that translocation appeared in mice even in long-term experiments.

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A PHAGE TYPING SYSTEM FOR *SALMONELLA INFANTIS*

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A phage typing method applying 9 type phages was elaborated to subdivide *Salmonella infantis*. Results are reported by the use of Farmer's mnemonic. Out of 4847 *S. infantis* strains, 4602 were of human and 245 of non-human origin. The strains were typable in 98.9%. Two phage types occurred more frequently than 20%, four phage types between 5 and 10%, seven phage types less than 5%, and twenty-eight phage types less than 1%. The strains originated from outbreaks in 28.7% and from sporadic cases in 71.3%. A total of 1320 strains examined for phage type was isolated from 4 field epidemics, 39 community outbreaks and 370 family infections. In the second version of the method two phages were substituted by two more effective ones. The phage typing method was suitable for epidemiological purposes. Inducing in vitro changes in phage types by lysogenization and plasmid acquisition, phage types 111, 113, 311, 313 and 343 changed to phage types 213, 243, 513 and 543 after lysogenization and phage types 311 and 543 to phage types 548, 565 and 885 due to plasmid acquisition.

Though *S. infantis* is one of the most significant and common *Salmonella* serotype throughout the world, a standardized, international phage typing method to subdivide this serotype is not available. Kasatiya et al. in 1979 [1] worked out a method to differentiate *S. infantis* strains in Canada. By the use of 9 phages (out of which 3 phages were isolated from sewage and 6 from human faeces) 546 strains were divided into 23 phage types; five frequent phage types were found (9.2–25.8%). In 14 different infections (a hospital outbreak, two field epidemics, a family infection and sporadic cases with more than one isolation), the phage types of strains that derived from the same source were identical. To differentiate 58 *Salmonella* serovars (serotypes) Gershman [2] developed a phage set consisting of 50 phages isolated from sewage. A total of 735 strains was characterized on the basis of 347 kinds of phage pattern. The 10 *S. infantis* strains belonged into four phage types. The method was modified [3] and by the help of 27 phages 1245 cultures were grouped on the basis of the 420 phage patterns observed.

Because of the increasing frequency of *S. infantis* in Hungary, it was desirable to elaborate a phage typing method to subdivide this serotype.

Materials and methods

Bacterial strains. Phage type was determined of a total of 4847 *S. infantis* strains isolated in Hungary between 1983 and 1985 (4602 human strains and 245 strains derived from animals, water and hygienic control examinations).

Type phages and propagating strains. Phages 1, 8, 10, 19, 21 and 38 (which lysed *S. infantis* strains) from Gershman's [2] phage-set were propagated on their original propagating strains and then adapted to *S. infantis* strains isolated from patients in Hungary (Table I/a).

On the basis of the lytic patterns obtained on *S. infantis* strains a phage-set was developed, containing 9 phages (a-i); this was the first version of the method. Since phage typing carried out in 1983 and 1984, revealed that phages *h* and *i* differentiated ineffectively, two more efficacious phages have been substituted for these. Instead of phage *h*, a phage isolated from a phage carrier *S. infantis* strain was used and instead of phage *i* phage *Salmonella* O1 was employed; this was the second version of the method. Table I/b shows the phages of the second version, the propagating strains and their properties.

Table I/a

Origin of S. infantis phages and their propagating strains
(Gershman's [2] phages and the first version of the present method)

Gershman		First version		
phages	propagating strains	phages	propagating strains*	
			designation	phage type
1	<i>S. heidelberg</i> 1	h	565	541
8	<i>S. thompson</i> 8	a	220	541
		f	518	211
10	<i>S. thompson</i> 10	b	736	211
		e	733	541
19	<i>S. newport</i> 19	i	996	215
21	<i>S. newport</i> 21	d	996	215
38	<i>S. cerro</i> 36	c	814	341
		g	428	885

* *S. infantis* strains

Table I/b

S. infantis phages and their propagating strains
(Second version of the present method)

Designation of phages		Propagating strains					
first	second	designation	phage type	lysogenic	colicinogenic	antibiotic resistance	plasmid size (Md)
version				properties			
a	1	220*	543	+	+	Sm	97
b	2	736*	213	+	—	—	—
c	3	814*	343	—	—	—	—
d	4	996*	213	+	—	—	—
e	5	733*	541	+	—	—	—
f	6	518*	211	+	—	—	2.0, 1.6, 1.2
g	7	428*	883	+	—	—	—
—	8	1343*	111	—	+	—	64
—	9	B76**	1***	—	—	—	—

* *S. infantis*

** *S. paratyphi-B*

*** According to Felix and Callow's [4] phage typing method

Strains and their properties to control the lytic spectra of the phages are listed in Table II.

Propagation of phages was carried out by the "cutting out" method, starting from one single plaque. To obtain bacteria-free preparation, the culture was passed through G5 glass filter. Phages were used in RTD (routine test dilution) determined by titration of the phage on the propagating strain and on the lytic-spectra control strains.

Determination of phage types. The 2 h broth culture of the strain to be typed was poured on an agar plate, and the excess was drawn off. After the culture had dried, type phages in routine test dilution (RTD) were spotted on the plate, incubated at 37 °C overnight and the lytic pattern was read.

Phage types were reported according to Farmer's [5] mnemonic (Table III).

Induction of lysogenic strains by mitomycin C. Mitomycin C (10 µg/ml) was added in 0.1 ml aliquots to 1.9 ml of the 2 h broth culture of the lysogenic strain (final concentration, 0.5 µg/ml). After standing for 30 min it was centrifuged (3000 rpm, 20 min) and to the sediment 2 ml prewarmed broth was added and incubated at 37 °C for 3 h. After centrifugation the supernatant was dropped on the surface of agar plates which were poured over with the broth cultures of the indicator strains and incubated overnight. Indicator strains: 736, 996, 518, 1343 (propagating strains); 613 (lytic-spectra control strain); 1062 (strain of phage type 113, lysogenic, plasmid-free); 1248 (strain of phage type 313, not lysogenic, resistant to Sm, coli-

Table II
Strains to control the lytic-spectra of *S. infantis* phages
(Second version of the method)

Designation of strain	Lytic pattern defined by type phages									Phage type	Lysogenic	Colicinogenic	Antibiotic resistance	Plasmid size (Md)
	1	2	3	4	5	6	7	8	9					
1343	+++			+++			+++			111	—	+	—	64
736	++—			+++			++—			213	+	—	—	—*
1574	++—	+		+++			+++			311	+	+	Ap	63
814	++—			+++			++—			343	—	—	—	—
635	++—			+++			++—			517	—	—	—	56
220	++—			+++			++—			543	+	+	Sm	97
613	++—			+++			++—			545	+	+	Tc Nm Km	72
428	—	—	—	—	—	—	++—			883	+	—	—	—

* The presence of plasmid was not demonstrated

Table III
Mnemonic for reporting phage types*

Designation	Lytic pattern		
1	+	+	+
2	+	+	—
3	+	—	+
4	—	+	+
5	+	—	—
6	—	+	—
7	—	—	+
8	—	—	—

For example: + — — — + + + + + = 541

* According to Farmer's [5] method

cinogenic, 72 Md plasmid); 152 (strain of phage type 111, not lysogenic, plasmid-free); 1197 (strain of phage type 543, lysogenic, resistant to Sm Cm Tc Ap Cot, plasmid-pattern 48, 16, 3.4 Md).

Lysogenization. The 2 h broth culture of the strain to be lysogenized was poured over an agar plate. The supernatant of the lysogenic strain after induction by mitomycin C was heated at 56°C for 30 min and spotted on the plate and incubated overnight at 37 °C.

The secondary colonies (probably lysogenic ones), grown on the surface of the lytic spot were streaked onto agar plates and subcultured three times. To control the lysogenic state of the colonies, they were tested (a) for immunity developed against the lysogenizing phage, (b) for the ability to release temperate phage from the colonies.

Determination of colicin type was carried out according to Frédéricq [6], Alwis and Thomlinson [7] and Horak [8].

Antibiotic sensitivity was determined to tetracycline (Tc), chloramphenicol (Cm), streptomycin (Sm), kanamycin (Km), neomycin (Nm), ampicillin (Ap), gentamicin (Gm) and co-trimoxazole (Cot), by the use of "Resistest" disks (Human Institute for Serobacteriological Production and Research, Budapest).

R-plasmid transfer was carried out by the broth method: the 2 h broth cultures of the donor and recipient strains were mixed 1:1 and added to 4.5 ml broth; after overnight incubation at 37 °C the cultures were diluted and 0.1 ml aliquots plated on selective media and incubated at 37 °C for 17 h. The colonies were subcultured on selective agar plates.

Incompatibility groups were determined as described [9–11].

Plasmid analysis. Plasmid DNA was prepared by the method of Kado and Liu [12], plasmid DNA was detected by electrophoresis in vertical agarose gels according to Meyers et al. [13]. Standard plasmids for molecular size estimation: R27 (Inc H) 112 Mdal; R1 (Inc FII) 62 Mdal; V517, 35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8, 1.4 Mdal [14].

Media. Oxoid nutrient broth No. 2 and agar were used for phage typing and propagation, sensitivity to antibiotics was tested on Mueller-Hinton agar.

Table IV

Phage type distribution of human *S. infantis* strains*
(Hungary, 1983–1985)

Phage type	Percentage of strains in year			Total
	1983	1984	1985	
111	0.8	1.0	7.2	2.5
211	26.4	16.6	25.2	22.4
214	0.5	3.0	6.4	2.9
241	7.4	8.6	4.2	7.1
244	2.1	2.5	1.6	2.1
311	11.2	2.4	2.2	5.5
341	8.7	7.1	4.8	7.1
344	1.7	1.4	0.2	1.2
511	3.1	3.7	1.7	3.0
514	0.5	0.4	0.3	0.4
541	24.5	33.3	31.6	29.7
544	6.9	13.8	6.6	9.5
685	0.7	0.4	0.2	0.4
885	1.6	2.9	4.5	2.8
888	1.0	1.2	1.0	1.1
Other (22–26 kinds)	2.9	1.7	2.3	2.3
Total	1695	1747	1160	4602

* According to the first version of the method

Results

Phage type distribution of human and non-human strains. Table IV shows the phage type distribution of 4602 human *S. infantis* strains isolated between 1983 and 1985. Phage typing was carried out according to the first version of the method. The strains were typable in 98.9%. Phage types 211 and 541 were more frequent than 20%. Four phage types, 241, 311, 341 and 544 occurred more frequently than 5%. The frequency of seven phage types varied between 1 and 5%, whereas twenty to twenty-eight different kinds of phage type occurred less than 1%. Comparing the yearly distribution of phage types, it was found that in 1983 the most frequent was phage type 211, followed by type 541, and type 311 was next in order. In 1984 and 1985 phage type 541 became the most frequent, 211 was the second, and the frequency of phage type 311 decreased from 11.2% to 2.4% and 2.2%, respectively. In 1984 the frequency of types 241 and 544 increased, but in 1985 it decreased. Phage type 111, which was rare in the first two years of examination became the third frequent phage type in 1985; phage types 214 and 885 occurred likewise more frequently in this year.

The phage type distribution of strains isolated from animals, food samples and in course of hygienic control examinations was similar to the human strains (Table V). The most frequent phage types were 541 and 211; the frequency of types 241, 341 and 544 was higher than 5%, six phage types (214,

Table V
*Phage type distribution of S. infantis strains of non-human origin**
(Hungary, 1983–1985)

Phage type	No. of strains in year			Total	
	1983	1984	1985	No.	%
211	4	40	23	67	27.3
214	4	1	1	6	2.5
241	2	5	6	13	5.3
244	2	—	4	6	2.5
311	2	3	5	10	4.1
341	5	8	2	15	6.1
511	2	7	1	10	4.1
541	15	38	25	78	31.8
544	2	8	6	16	6.5
885	3	1	5	9	3.7
888	1	—	2	3	1.2
Other	1	8	3	12	4.9
Total	43	119	83	245	100.0

* According to the first version of the method. The strains were isolated from animals, food samples, water and in the course of hygienic control examinations.

Table VI

*Geographic distribution of S. infantis phage types derived from field epidemics**
(Hungary, 1983-1985)

Location (county)	Total No. of		Phage types							
			211		214		241		541	
			No. of		No. of		No. of		No. of	
	epidem- ics	strains	epidem- ics	strains	epidem- ics	strains	epidem- ics	strains	epidem- ics	strains
Csongrád	2	57	1	28		20			1	9
Pest	1	4					1	4		
Szolnok	1	8	1	8						
Total	4	69	2	36		20	1	4	1	9

* Phage typing was carried out according to the first version of the method

244, 311, 511, 885, 888) occurred in 1 to 5% and 8 to 12 kinds of rare phage type were found in less than 1%.

Phage type of strains derived from outbreaks. The epidemiological application of *S. infantis* phage typing is demonstrated in Tables VI, VII and VIII. Phage type was determined of a total of 1320 strains originating from 4 field epidemics, 39 community outbreaks and 370 family infections. The 69 strains isolated from the four field epidemics (Table VI) belonged to phage types

Table VII

Geographic distribution of S. infantis phage
(Hungary,

Location (county or Budapest)	Total No. of		Phage					
			211		241		311	
			No. of		No. of		No. of	
	out- breaks	strains	out- breaks	strains	out- break	strains	out- breaks	strains
Bács-Kiskun	1	3						
Békés	2	54						
Csongrád	6	50	1	22	1	15	1	3
Fejér	5	25			1	3	1	5
Győr	2	15						
Heves	6	54	4	31	2	23		
Pest	6	81	1	14	2	20		
Somogy	5	78	2	61				
Tolna	1	18						
Vas	1	14	1	14				
Budapest	4	16						
Total	39	408	9	142	6	61	2	8

* Phage typing was carried out according to the first version of the method

211, 211 and 214, 241 and 541. In the field epidemics the source of infection was contaminated food (brawn, chicken, meat, mixed salad). In one of the epidemics (in Csongrád county) the strains belonged to two kinds of phage types.

Phage type distribution of the strains isolated from community outbreaks is presented in Table VII. The community outbreaks took place in nurseries, kindergartens, schools, weddings, old people's home. More than half [22] of the 39 community outbreaks were caused by strains of phage types 541 and 211. Strains of phage type 241 were found in 6 outbreaks, which took place in four counties (Csongrád, Fejér, Heves, Pest). An outbreak in a children's community in Pest county was caused by strains of phage type 511, resistant to Tc, Nm Km.

Phage types of the strains causing family infections are shown in Table VIII. Phage type was determined of a total of 843 strains isolated from 370 household infections between 1983 and 1985. The majority of the strains derived from family infections were isolated in counties Pest, Csongrád, Heves, Szolnok and Vas. Phage types 541 and 211 were the most frequent also among the strains derived from family infections. The majority of the family outbreaks were caused by strains of phage type 211 in Csongrád, Heves, Szolnok, Tolna and Veszprém. Household infections were frequently caused by strains of type 111 in Nógrád and Szolnok, strains of phage type 544 in Hajdú and

*types derived from community outbreaks**
1983-1985)

type									
341		511		541		544		885	
No. of		No. of		No. of		No. of		No. of	
outbreaks	strains	outbreaks	strains	outbreaks	strains	outbreaks	strains	outbreaks	strains
				1	3				
				2	54				
1	4			2	6				
				1	2			2	15
1	12					1	3		
1	3	1	41	1	3				
				3	17				
				1	18				
1	2			2	12	1	2		
4	21	1	41	13	115	2	5	2	15

Table VIII
Geographic distribution of S. infantis phage
(Hungary,

Location (county or Budapest)	Total No. of		Phage types/No.			
	outbreaks	strains	111	211	214	241
Baranya	2	7				
Békés	3	12		4		
Borsod-A.-Z.	6	28		4		
Csongrád	23	88		30	6	6
Fejér	6	29		4		
Győr-Sopron	13	54		9	1	2
Hajdú-Bihar	7	44	4			
Heves	32	81	1	63		4
Komárom	2	6				
Nógrád	7	18	9	6	1	
Pest	11	112	1	19	3	19
Somogy	2	46		6		7
Szabolcs-Szatmár	19	48		5	3	6
Szolnok	16	81	13	23	1	2
Tolna	11	29		10		2
Vas	30	71	2	6	7	2
Veszprém	10	27	1	13		2
Zala	21	49		12		
Budapest	4	13				
Total	370	843	31	214	22	52

* Phage typing was carried out according to the first version of the method

Szabolcs, strains of phage type 544 and 341 in Győr-Sopron and strains of phage type 341 and 241 in Pest.

Table IX presents the phage type distribution of 4602 strains. The strains were derived in 28.7% from epidemics and in 71.3% from sporadic cases, i.e. the ratio of the number of epidemic: sporadic strains was 1:2.5. As regards the incidence of different phage types, this ratio varied significantly: for example the ratio of epidemic and sporadic strains for phage type 311 was 1:3.8, for phage type 544 1:4.2, for phage type 244 1:7.0. In epidemics registered between 1983 and 1985 strains of phage types 211, 241 and 541 were the most frequent whereas the incidence of phage type 211 and 241 strains was much lower than the average (1:1.6; 1:1.8). For phage type 541 the ratio was nearly to the average (1:2.4).

The second version of the phage typing method. On the basis of phage typing of about three and a half thousand strains the second version of the method was elaborated by omitting two ineffective phages and by the addition of two new phages. The phage type of 1160 strains isolated in 1985 was determined parallel according to the first and second version. Table X shows the phage type distribution obtained by the two versions. As it had been expected, the second version was more effective, since the majority of the two frequent phage types (211 and 541) could be divided into further two and three types,

types derived from family outbreaks*
1983-1985)

of strains										
244	311	341	344	511	541	544	685	885	888	Other
	7				6					
	4	2			14	1				
	19	3	2		9	6				3
	6	6	3		12			7		
1	2	7	4		17	8		1	1	1
		2			28	10				
2					3	6				2
					6					
			1		1					
3	2	20		6	28	6		4		1
		4			22	7				
2					16	12	2	2		
4	2	3	4	4	20	5				
	3		2		6	6				
		6		4	40	4				
					11					
		6		2	20	7		2		
					13					
12	45	59	16	16	272	78	2	16	1	7

respectively. Subdivision according to the second version was especially useful for the original type 211, whereas in the case of phage type 541 the subdivision was less effective, because the majority of the strains belonged to phage type 543, though, 3 other phage types were detected.

In vitro changes of phage types due to lysogenization by type phages. Changes of phage types following lysogenization by type phages were examined. Strains of phage types 111, 211, 343 and 543 were lysogenized by type phages 1, 2, 3, 5, 6, 7 and 8. The changes in phage sensitivity of the strains are shown in Table XI. After lysogenization, besides the lysogenizing phage, immunity was observed to other phages, too. Out of the 12 new phage types obtained in this manner, 8 occurred in the human material.

*Changes of phage types following lysogenization by temperate phages liberated from lysogenic *S. infantis* strains. Examination of lysogenic state of *S. infantis*.* Lysogenic state examination of 93 *S. infantis* strains showed that they belonged to 25 kinds of phage type. The strains were lysogenic in 63.4%. Lysogeny was frequent in the case of strains of phage type 543, 547, 548 and 883, among the strains of phage type 111, 211, 241 lysogenic ones were rare or absent.

Changes of phage types were observed after lysogenizing strains of phage type 111, 113, 311, 313 and 343 by temperate phages liberated from

Table IX
Percentage distribution of phage types of S. infantis strains derived from outbreaks and sporadic cases (Hungary, 1983-1985)

Location (county or Budapest)	Total No. of strains		Phage type											
			111		211		214		241		244		311	
			0	S	0	S	0	S	0	S	0	S	0	S
Baranya	7	13	—	—	—	100.0	—	—	—	100.0	—	—	87.5	12.5
Bács-Kiskun	3	3	—	—	—	—	—	—	—	100.0	—	—	—	—
Békés	67	133	—	100.0	11.8	88.2	—	100.0	—	100.0	—	100.0	—	—
Borsod-A.-Z.	33	173	—	100.0	20.0	80.0	—	100.0	—	100.0	—	100.0	25.0	75.0
Csongrád	189	293	—	100.0	64.0	36.0	86.7	13.3	60.0	40.0	—	100.0	25.3	74.7
Fejér	65	182	—	100.0	13.8	86.2	—	100.0	42.9	57.1	—	100.0	34.4	65.6
Győr	68	141	—	100.0	33.3	66.7	25.0	75.0	16.7	83.3	100.0	—	18.2	81.8
Hajdú-Bihar	40	160	57.1	42.9	—	100.0	—	100.0	—	100.0	—	100.0	—	100.0
Heves	129	318	11.1	88.9	38.8	61.2	—	100.0	52.9	47.1	11.1	88.9	—	100.0
Komárom	6	5	—	—	—	—	—	—	—	—	—	—	—	—
Nógrád	18	80	75.0	25.0	20.7	79.3	20.0	80.0	—	100.0	—	100.0	—	100.0
Pest	197	411	33.3	66.7	35.5	64.5	27.3	72.7	55.8	44.2	18.8	81.2	7.7	92.3
Somogy	124	118	—	100.0	89.3	10.7	—	100.0	100.0	—	—	—	—	100.0
Szabolcs-Sz.	48	196	—	100.0	13.9	86.1	100.0	—	20.0	80.0	33.3	66.7	—	100.0
Szolnok	89	214	61.9	38.1	35.2	64.8	8.3	91.7	20.0	80.0	80.0	20.0	20.0	80.0
Tolna	47	134	—	100.0	34.5	65.5	—	100.0	20.0	80.0	—	100.0	30.0	70.0
Vas	85	192	100.0	—	47.6	52.4	41.2	58.8	14.3	85.7	—	100.0	—	100.0
Veszprém	27	81	14.3	85.7	48.1	51.9	—	100.0	20.0	80.0	—	100.0	—	100.0
Zala	49	103	—	100.0	44.4	55.6	—	100.0	—	100.0	—	100.0	—	100.0
Budapest	29	332	—	100.0	—	100.0	—	100.0	—	100.0	—	100.0	—	100.0
Total No. of strains	1320	3282	31	85	392	638	42	93	117	269	12	85	53	202

Location (county or Budapest)	Total No. of strains		Phage type													
			341		344		511		541		544		885		Other	
	0	S	0	S	0	S	0	S	0	S	0	S	0	S	0	S
Baranya	7	13	—	—	—	—	—	100.0	—	100.0	—	100.0	—	—	—	100.0
Bács-Kiskun	3	3	—	100.0	—	—	—	100.0	100.0	—	—	—	—	—	—	—
Békés	67	133	66.7	33.3	—	—	—	100.0	53.6	46.4	5.9	94.1	—	100.0	—	100.0
Borsod-A.-Z.	33	173	12.5	87.5	25.0	75.0	—	100.0	25.9	74.1	18.8	81.2	—	100.0	—	100.0
Csongrád	189	293	15.6	84.4	23.1	76.9	—	100.0	36.9	63.1	—	100.0	—	100.0	18.8	81.2
Fejér	65	182	—	100.0	—	100.0	—	100.0	17.7	82.3	68.8	31.2	66.7	33.3	—	100.0
Győr	68	141	82.6	17.4	100.0	—	—	100.0	24.6	75.4	37.0	63.0	16.7	83.3	14.3	85.7
Hajdú-Bihar	40	160	28.6	71.4	—	—	—	100.0	25.7	74.3	42.9	57.1	—	100.0	—	100.0
Heves	129	318	—	100.0	—	100.0	—	100.0	6.3	93.7	—	100.0	—	100.0	11.1	88.9
Komárom	6	5	—	100.0	—	—	—	—	100.0	—	—	100.0	—	—	—	100.0
Nógrád	18	80	—	100.0	100.0	—	—	100.0	4.5	95.5	—	100.0	—	100.0	—	100.0
Pest	197	411	29.1	70.9	—	100.0	79.7	20.3	22.3	77.7	9.8	90.2	26.7	73.3	4.3	95.7
Somogy	124	118	57.1	42.9	—	100.0	—	100.0	39.0	61.0	31.8	68.2	—	100.0	—	100.0
Szabolcs-Sz.	48	196	—	100.0	—	—	—	100.0	16.7	83.3	32.4	67.6	28.6	71.4	16.7	83.3
Szolnok	89	214	23.1	76.9	100.0	—	50.0	50.0	26.7	73.3	19.2	80.8	—	100.0	—	100.0
Tolna	47	134	—	100.0	100.0	—	—	100.0	32.4	67.6	23.1	76.9	—	100.0	—	100.0
Vas	85	192	26.1	73.9	—	100.0	36.4	63.6	33.6	66.4	20.0	80.0	—	100.0	—	100.0
Veszprém	27	81	—	100.0	—	—	—	100.0	34.4	65.6	—	100.0	—	100.0	—	100.0
Zala	49	103	40.0	60.0	—	100.0	50.0	50.0	39.2	60.8	25.9	74.1	33.3	66.7	—	100.0
Budapest	29	332	14.3	85.7	—	100.0	—	100.0	22.3	77.7	4.9	95.1	—	100.0	—	100.0
Total No. of strains	1320	3282	80	247	16	38	57	81	396	970	83	353	31	98	10	183

0 = strains isolated from outbreaks
S = strains isolated from sporadic cases

Table X

Phage type distribution of human *S. infantis* strains according to the first and second version of the method
(Hungary, 1985)

First version			Second version			First version			Second version		
Phage type	Strain		Phage type	Strain		Phage type	Strain		Phage type	Strain	
	No.	%		No.	%		No.	%		No.	%
111	84	7.2	111	54	4.6	514	4	0.3	516	1	0.1
			113	30	2.6				517	2	0.2
211	292	25.2	211	90	7.8				518	1	0.1
			213	201	17.3	541	367	31.6	541	17	1.4
			215	1	0.1				542	2	0.2
214	74	6.4	214	15	1.3				543	322	27.8
			217	59	5.1				545	26	2.2
241	49	4.2	241	21	1.8	544	77	6.6	544	3	0.3
			243	28	2.4				547	67	5.7
244	18	1.6	244	8	0.7				548	7	0.6
			247	10	0.9	685	2	0.2	683	2	0.2
311	25	2.2	311	5	0.4	885	52	4.5	883	44	3.8
			313	20	1.7				885	8	0.7
341	56	4.8	341	1	0.1	888	12	1.1	887	9	0.8
			343	50	4.3				888	3	0.3
			345	5	0.4						
344	2	0.2	344	2	0.2	Other	26	2.2	Other	26	2.2
511	20	1.7	511	3	0.3						
			512	1	0.1						
			513	16	1.3	Total	1160	100.0		1160	100.0

Table XI

Changes of phage types following lysogenization by *S. infantis* type phages

Original phage type*	Lysogenizing type phage	Sensitivity was lost to type phages	New phage type*
211	1	1, 5, 7, 8	637
343	1	1, 5, 6	783**
211	2	2, 4, 7, 8	547**
111	3	3, 7, 8	217**
343	3	3	543**
111	5	1, 5	431
343	5	1, 5, 6, 7	787
543	5	1, 5, 6, 7	887**
111	6	1, 3, 4, 5, 6, 7, 8	687**
543	6	1, 5, 6	883**
111	7	1, 3, 5, 7, 8	637
111	8	3, 7, 8	217**

* Phage types were determined according to the second version of the method

** Phage types occurred also in vivo

Table XII*Changes of phage types after lysogenization by temperate S. infantis phages*

Phage type* before lysogenization	Phage type of the strains carrying the temperate phages	Sensitivity was lost to type phages	Phage type* after lysogenization
111	213, 243, 247, 513, 543, 547, 548	3, 8	213
113	543	3, 4	243
113	547	3	213
311	213, 541, 543, 547, 883	3, 8	513
313	213, 243, 247, 513, 543, 547, 548	3	513
343	211, 213, 543, 883	3	543

* Phage types were determined according to the second version of the method

Table XIII*Changes of phage types in S. infantis strains due to plasmid acquisition*

Original phage type*	Characters of acquired plasmid				Restricted phages	New phage type*
	antibiotic resistance	Inc.	col.	size (M dal)		
543	Tc Nm Km	I α	Ib	72	6, 9	565
311	Tc Nm Km	I α	Ib	72	3, 4, 7, 8, 9	548
311	Nm Km	I γ	Ib	76	1, 3, 4, 5, 6, 8, 9	885

* Phage types were determined according to the second version of the method

lysogenic *S. infantis* strains belonging to different phage types. The results are presented in Table XII. Lysogenization resulted in a loss of sensitivity to type phages 3, 4 and 8. Phage types changed from 111 to 213 and from 113 to 213 and 243. Phage types 513 and 543 developed from types 311, 313 and 343.

Changes of phage types due to plasmid acquisition were also examined (Table XIII). A plasmid derived from a *S. infantis* strain (Inc I α , 72 Md, controlling Tc Nm Km resistances and Ib colicin production) was transferred to strains of phage type 543 and 311. Phage restrictive effect being due to plasmid acquisition, sensitivity was lost to type phages 6, 9 and 3, 4, 7, 8, 9, and the phage types changed to 565 and 548, respectively. Phage type 311 changed to 885 after the acquisition of another plasmid (Inc group I γ , 76 Md, controlling resistance to Nm Km and colicin Ib production). Phage type 885 was found in the course of phage typing examinations, too.

Discussion

S. infantis has become increasingly prevalent throughout the world; in Europe it is now falling between the 3rd and 9th rank among the *Salmonella* serotypes. It was the 3rd in Italy and in the Netherlands, the 4th in Finland,

the 5th in Austria and England, the 6th in Poland, Norway, Sweden and Yugoslavia, the 7th in France and Scotland, the 9th in Belgium. *S. infantis* occupied the 7th rank among the salmonellae in the USA [15]. In Hungary, according to data of the National Salmonella Centre [16], *S. infantis* was isolated from 6560 persons between 1983 and 1985 and in this period *S. infantis* became the 2nd and 3rd most common serotype. Thus, there was an obvious need for a phage typing method allowing a further differentiation of *S. infantis* strains. Gershman's methods, although using numerous phages, distinguished only four groups among the examined *S. infantis* strains. By the use of a phage set developed in the present work, the serotype could be classified into 60 phage types and 99.7% of the isolates were typable. The properties of the propagating and lytic-spectra control strains were examined to assure the stability of the phages and phage types. Accordingly, this method proved to be suitable for the subdivision and epidemiological tracing of *S. infantis* strains. For reporting phage types, Farmer's mnemonic seems to be better than a phage typing scheme because it gives a possibility to designate new phage types which may appear after a change in the bacterial population. The disadvantage of the method is that the differences in the lytic degree are not expressed in the phage types.

Lysogenization by type phages resulted immunity not only to the lysogenizing phages, but also to other phages; cross-immunity was observed between phages 1, 5 and 6 as well as between phages 3 and 7. The relationship between phages 1 and 6, and 3 and 7 is evident, because they are host-modified derivatives of one and the same phage, but there is no such relation between phages 1 and 5.

Conclusion may be drawn from the in vitro changes of *S. infantis* phage types that had developed as a consequence of lysogenization and plasmid acquisition. Changes of phage types due to R-plasmids were reported in *Salmonella typhi* [17-20], *Salmonella typhi-murium* [21-25], *Salmonella panama* [26, 27] and *Shigella* [28-32]. It is most probable that in the evolution of the variety of phage types such mechanisms played an important part.

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A SIMPLE IDENTIFICATION SYSTEM FOR RAPIDLY GROWING MYCOBACTERIA

(A NOTE)

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Based on a ten-year experience, a simple identification system for rapidly growing mycobacteria has been presented for clinical laboratories not specialized in this work. The system consists of 12 tests (8 media and 2 micromethods).

Within the genus *Mycobacterium*, the importance of rapidly growing mycobacteria has greatly increased in recent years and more than ten new species have been described [1–7]. For the identification laborious biochemical, cultural and immunological methods have been used. In contrast with these time consuming techniques, based on experience of our laboratory concerned with the identification of mycobacteria since 10 years, a simple identification system of rapidly growing mycobacteria is presented for clinical laboratories which are not specialized for this work.

Materials and methods

Bacterial strains. A total of 84 strains of rapidly growing mycobacteria was employed in this study. They were partly received from culture collections, partly isolated in this laboratory from a variety of sources (surface water, soil, urine, pus and sputum). The strains were identified in this laboratory by methods described previously [8–11].

Tests and media. The tests and media used are listed in Table 1.

Inoculation of media. A bacterial suspension (10 mg wet weight per ml) was prepared from a 3 to 5 day-old Ogawa egg medium slant culture. One loopful sample (0.01 ml) of the suspension was inoculated onto each medium, and the media were stoppered by rubber cap with a 3 mm cut-line in its bottom and incubated at 37 °C in the dark, except for one tube of the control Ogawa egg medium, which was incubated at 37 °C exposed to light (a 40-W tungsten bulb, 30 cm).

Reading of results. After incubation for 3 days, the growth on the control Ogawa egg medium incubated in the dark, was observed. If abundant membranous growth occurred after 3 days, the results of all tests were read after incubation for 7 days. If no growth or only scanty growth occurred after 3 days, the results of all tests were read after incubation for 14 days.

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Table I
Tests and media for the simple identification system

Tests	Medium
1. Growth after 3 days	Ogawa egg medium (incubated in the dark)
2. Tolerance to 0.2% picric acid	A modified Sauton agar medium containing 0.2% picric acid [12]
3. Colony pigmentation in the dark	Ogawa egg medium (incubated in the dark) [13]
4. ONPG (beta-galactosidase)	Substrate: 2-naphthyl-beta-D-galactopyranoside pH 5.4 [10]
5. Nitrate reduction (24 h)	Basal medium with NaNO ₃ and Erlich's aldehyde reagent [14]
6. Resistance to ethambutol	Ogawa egg medium containing ethambutol (5 µg/ml) [15]
7. Tween hydrolysis (after 7 days)	Basal medium with Tween 80 (0.5%) and neutral red as indicator [16]
8. Resistance to hydroxylamine	Ogawa egg medium containing hydroxylamine monochlorhydrate (0.5 mg/ml) [17]
9. Degradation of salicylate	Ogawa egg medium containing 0.2% sodium p-aminosalicylate [18]
10. Acid phosphatase	Substrate: 2-naphthyl-phosphate pH 5.4 [10]
11. Resistance to cycloheximide (actions)	Ogawa egg medium containing cycloheximide (0.5 mg/ml) [9]
12. Gelatin hydrolysis (after 7 days)	Photographic film method [11]

Results and discussion

The results of the tests are shown in Table II. From these data, an identification table was constructed (Table III). All results obtained with this identification system were read after incubation at 37°C for 7 days. All 84 strains could be identified by using this system. The main differential characters are as follows.

(A) Non-pigmented strains

M. fortuitum is the only species which hydrolyse gelatin;
M. chelonae subs. *chelonae* fails to grow in Sauton agar with picric acid;
M. chelonae subs. *abscessus* differs from *M. chelonae* subs. *chelonae* in acid phosphatase and tolerance to picric acid;
M. smegmatis differs from *M. agri* in resistance to ethambutol;
M. chitae differs from *M. agri* in nitrate reduction (24 h).

(B) Pigmented strains

M. rhodesiae fails to grow in Sauton agar with picric acid and hydrolyse gelatin after 4 days;

M. thermoresistibile hydrolyse gelatin after 48 h;
M. phlei differs from *M. valentiae* in acid phosphatase;
M. obuense decomposes p-aminosalicylate;
M. neoaurum, *M. tokaiense* and *M. obuense* grow in Ogawa egg medium with 0.5 µg/ml ethambutol;
M. vaccae and *M. parafortuitum* differ in Tween hydrolysis (7 days) and acid phosphatase;
M. flavescens and *M. aurum* differ in Tween hydrolysis (7 days);
M. aichiense differs from *M. chubuense* in acid phosphatase;
M. duvalii differs from *M. gilvum* in resistance to actidione; both species differ from *M. gadium* in tolerance, to picric acid.

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Table II
 Characters of rapidly growing mycobacteria

Organisms	Number of strains	Growth after 3 days	Tolerance to picric acid	Colony pigmentation in dark	beta-Galactosidase	Nitrate reduction (24 h)	Resistance to ethambutol	Tween hydrolysis (7 days)	Resistance hydroxylamine	Degradation of PAS	Acid phosphatase	Resistance to actidione	Gelatin hydrolysis
Number of strains showing positive reaction													
<i>M. smegmatis</i>	5	5	5	0	2	5	1	0	0	0	4	5	0
<i>M. fortuitum</i>	12	12	12	0	0	12	12	0	12	10	12	12	12
<i>M. chelonae</i> subsp. <i>chelonae</i>	3	3	0	0	3	0	3	0	3	3	3	3	0
<i>M. chelonae</i> subsp. <i>abscessus</i>	5	5	5	0	5	0	5	0	5	5	0	0	0
<i>M. chitae</i>	2	2	2	0	0	2	0	0	2	0	2	0	0
<i>M. agri</i>	1	1	1	0	0	0	0	0	0	0	0	1	0
<i>M. vaccae</i>	1	1	1	1	0	0	0	1	0	0	0	0	0
<i>M. diernhoferi</i>	1	1	1	1	1	0	0	0	0	0	1	1	0
<i>M. phlei</i>	15	15	15	15	15	15	0	15	0	0	15	15	0
<i>M. gilvum</i>	5	5	5	5	0	5	0	0	0	0	0	0	0
<i>M. duvalii</i>	4	4	4	4	0	4	0	0	0	0	0	4	0
<i>M. rhodesiae</i>	3	3	0	3	0	0	0	3	0	0	0	3	3
<i>M. gadium</i>	1	1	0	1	0	1	0	0	0	0	0	0	0
<i>M. flavescens</i>	2	2	2	2	0	2	0	2	0	0	0	0	0
<i>M. aurum</i>	6	6	6	6	0	0	0	0	0	0	0	0	0
<i>M. parafortuitum</i>	9	9	9	8	0	2	0	0	0	0	9	9	0
<i>M. valentiae</i>	5	5	5	5	5	5	0	5	0	0	0	5	0
<i>M. neoaurum</i>	1	1	1	1	0	0	1	1	0	0	0	1	0
<i>M. chubuense</i>	1	1	1	1	0	1	0	1	0	0	0	1	0
<i>M. aichiense</i>	1	1	1	1	0	0	0	1	0	0	1	1	0
<i>M. tokaiense</i>	1	1	1	1	0	0	1	1	0	0	0	0	0

* All results were read after incubation at 37 °C for 7 days

Table III
Identification table for rapidly growing mycobacteria

Organisms	Growth after 3 days	Tolerance picric acid	Colony pigmentation dark	beta-Galactosidase	Nitrate reduction (24 h)	Resistance ethanol-butol	Tween hydrolysis (7 days)	Resistance hydroxyl-amine	Degradation of PAS	Acid phosphatase	Resistance to acti-dione	Gelatin hydrolysis
<i>M. flavescens</i>	+	+	+	-	+	-	+	-	-	-	+	+
<i>M. thermoresistibile</i>	+	+	+	-	+	-	+	-	-	+	+	+
<i>M. phlei</i>	+	+	+	+	+	-	+	-	-	-	+	-
<i>M. vaccae</i>	+	+	+	-	-	-	+	-	-	-	-	-
<i>M. diernhoferi</i>	+	+	+	+	-	-	-	-	-	+	+	-
<i>M. parafortuitum</i>	+	+	±	-	±	-	-	-	-	+	+	-
<i>M. aurum</i>	+	+	+	-	-	-	-	-	-	-	-	-
<i>M. obuense</i>	+	+	+	-	-	+	+	-	+	-	+	-
<i>M. rhodesiae</i>	+	-	+	-	-	-	+	-	-	+	+	-
<i>M. neoaurum</i>	+	+	+	-	±	+	+	-	-	+	+	-
<i>M. aichiense</i>	+	+	+	-	-	-	+	-	-	+	+	-
<i>M. chubuense</i>	+	+	+	-	±	-	+	-	-	-	+	-
<i>M. tokaiense</i>	+	+	+	-	-	+	+	-	-	-	-	-
<i>M. duvalii</i>	+	+	+	-	+	-	-	-	-	±	+	-
<i>M. gilvum</i>	+	+	+	-	±	-	+	-	-	±	-	-
<i>M. gadium</i>	+	-	+	-	+	-	-	-	-	±	-	-
<i>M. valentiae</i>	+	+	+	+	+	-	+	-	-	-	+	-
<i>M. smegmatis</i>	+	+	-	+	±	+	±	-	-	-	+	-
<i>M. fortuitum</i>	+	+	-	-	±	+	-	+	±	+	+	+
<i>M. chelonae</i> subsp. <i>chelonae</i>	+	-	-	+	-	+	-	+	+	+	+	-
<i>M. chelonae</i> subsp. <i>abscessus</i>	+	+	-	+	-	+	-	+	+	-	-	-
<i>M. chitae</i>	+	+	-	-	+	-	-	+	-	+	-	-
<i>M. agri</i>	+	+	-	-	-	-	-	-	-	-	+	-

+: 95-100% positive
-: 95-100% negative

±: more than 50% positive
±: more than 50% negative

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BIOTYPING OF *SHIGELLA SONNEI*

(A NOTE)

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(Received April 9, 1987)

One thousand and fifty-nine *Shigella sonnei* strains were submitted to biotyping and colicine typing. Simultaneous use of biotyping and colicine typing may be considered of value in epidemiological tracing.

Biotyping is one of several methods used for identification of *Shigella sonnei* strains. In this work we present our experience with biotyping as compared with colicine typing.

Materials and methods

The *S. sonnei* strains were obtained from the Public Health Laboratory, Belgrade and from the Laboratory of the Department for Infectious Diseases, Belgrade.

Biotyping was performed by using the method of Solodovnikov et al. [1].

For colicine typing a modified version of the Abbott and Shannon method [2] was used. Nine indicators used for typing (*S. sonnei* 56, 17, 56/56, 2, R6, 2/7, R5, *Shigella dysenteriae* 2-M19 and *Escherichia coli* Row) were provided by the Dysentery Reference Laboratory, London.

Results and discussion

The results of simultaneous colicine typing and biotyping of *S. sonnei* strains are presented in Table I. By the use of colicine typing 59.2% of *S. sonnei* strains were differentiated into 10 types, 4.7% of strains belonged to unclassified types and 36.1% could not be typed. Out of the typable strains 45.1% belonged to types 3 and 4.

By biotyping all but five strains were classified into seven different types. However, type Ia accounted for 83.3% of all typable strains. Simultaneous use of two methods made differentiation of the strains better.

The reliability of biotyping results was assessed by (i) typing strains from serial isolations of one individual during clinical illness and convalescence and (ii) typing strains related epidemiologically.

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Table I
Biotyping and colicine typing of S. sonnei strains

Biotype	Colicine type												
	ut	2	3	3A	4	5	6	8	11	14	15	nc	Total
Ia	323	27	286	9	123	4	27	5	22	4	14	34	878
Ib	17	1	31	—	11	—	1	—	1	—	—	4	66
IIg	18	—	7	2	10	—	16	—	5	1	1	6	66
IIe	4	—	—	—	—	—	—	—	—	—	—	5	9
IIIc	1	—	—	—	—	—	1	—	—	—	—	—	2
IIId	15	—	4	1	3	—	7	—	—	—	—	1	31
IVf	1	—	1	—	—	—	—	—	—	—	—	—	2
ut	3	—	—	—	2	—	—	—	—	—	—	—	5
Total	382	28	329	12	149	4	52	5	28	5	15	50	1059

ut = untypable strains

nt = unclassified type

Table II
Variation in biotypes and colicine types of S. sonnei strains isolated from ten excretors

Excretors	Types on repeated isolation	
	biotype	colicine type
1	IIId/IIIc	12/6
2	IIg/IIId	nc/ut
3	Ia/IIg/IIg	6/4/4
4	Ia/Ia	nc/11
5	Ia/Ia	3A/ut
6	Ia/Ia	ut/4
7	Ia/Ia/Ia	ut/6/ut
8	IIg/Ia	nc/nc
9	IIIc/Ia	ut/ut
10	IIId/IIId/IIIa/IIId/IIId	6/6/6/6/6

ut = untypable strain

nc = unclassified type

Isolates from 40 excretors of *S. sonnei* were typed. In the majority of excretors *S. sonnei* was shown in two (31 patients) and three (8 patients) successive isolations. One patient was represented by five isolates.

Thirty persons excreted the same colicine and biotype on repeated examinations, whereas ten others excreted more than one type (Table II). Three of the latter showed variation in both colicine and biotype, three only in biotype and the remaining four only in colicine type.

Table III

Variation in biotypes and colicine types of S. sonnei strains isolated during four epidemics

Epidemic	Types isolated	
	biotypes	colicine types
1	IIg/Ia/Ia	nc/4/4
2	IIIId/Ia	ut/nc
3	Ia/IIg/Ia/Ia/Ia	5/ut/4/4/4
4	Ia/Ia/Ia	nc/3/3

ut = untypable strain

nc = unclassified type

Each of 12 epidemics of *S. sonnei*, involving 2 to 91 patients, was characterized by a uniform colicine and biotype. In 4 other epidemics the colicine and biotypes varied (Table III).

It may be assumed that biotyping, as a method for epidemiological studies of *S. sonnei*, is as reliable as colicine typing. However, biotyping cannot be proposed as a single typing method because of its poor differential value, but can be used as an additional means of epidemiological tracing. It would appear that nonuniformity of *S. sonnei* types isolated from an individual or during an epidemic may be better explained when two typing methods are used. When simultaneous changes occur in both colicine type and biotype, a multiple infection, superinfection or, in the case of an epidemic, the existence of more than one source of infection, seems to be the most acceptable explanation for nonuniformity. When only one of the types changes, the possibility of in vivo or in vitro variation in colicine production or biochemical activity may be considered.

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CONTENTS

Antibiotic Effects on Growth and Heterocyst Differentiation of the Cyanobacterium <i>Nostoc muscorum</i> Pattanaiik, U., Singh, P. K.	81
Selective Quantitative Determination of Tobramycin from Fermentation Broth Kiss, Gy. B., Széll, V., Ambrus, G., Ott, I.	89
Comparative Effects of Synthetic Insecticides — Endosulfan, Phosalone and Permethrin — on <i>Chlamydomonas reinhardtii</i> Algal Cells Gandhi, S. R., Kulkarni, S. B., Netrawali, M. S.	93
Utilization of Organic Substrates by two Filamentous Cyanobacteria Under Various Growth Conditions Adhikary, S. P., Sahu, J. K.	101
Effects of Hormones on the Multiplication of the Heterotrichous Protozoon <i>Blepharisma undulans</i> Stein Kovács, P., Csaba, G.	107
Determination of Phage Type, Colicin Type and Plasmid Profile in <i>Shigella sonnei</i> Tóth, I., Hajnal, A.	115
 TENTH CONGRESS OF THE HUNGARIAN SOCIETY OF MICROBIOLOGY	
Symposium I. Biotechnology and Industrial Microbiology	123
Symposium II. Biology of Interferon System	134
Symposium III. Latent and Persistent Viral Infections	148
Agricultural and Food Microbiology	151
Bacteriology and Parasitology	170
Immunology	192
Mycology	199
Virology	207

ANTIBIOTIC EFFECTS ON GROWTH AND HETEROCYST DIFFERENTIATION OF THE CYANOBACTERIUM *NOSTOC MUSCORUM*

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In a medium free of combined nitrogen, 1.0–6.0 $\mu\text{g/ml}$ chloramphenicol gradually decreased the growth of the nitrogen-fixing cyanobacterium *Nostoc muscorum*; at 2.0 $\mu\text{g/ml}$ the culture appeared yellowish, heterocyst frequency was not evident up to 3.0 $\mu\text{g/ml}$, whereas 4.0 $\mu\text{g/ml}$ suppressed heterocyst differentiation. Lower concentrations (0.0125–0.75 $\mu\text{g/ml}$) of rifampicin suppressed the growth but had no significant effect on heterocyst frequency in liquid medium, whereas on solid medium 0.02 $\mu\text{g/ml}$ produced chains of heterocysts in filaments. The growth and heterocyst frequency declined gradually with increasing doses of puromycin and 6.0 $\mu\text{g/ml}$ suppressed the heterocyst differentiation. Actinomycin-D did not affect significantly growth and heterocyst frequency even at 10.0 and 20.0 $\mu\text{g/ml}$.

Antibiotics are known to block various metabolic pathways in algal system and this has been employed in detection of the metabolic pattern of blue-green algae (cyanobacteria). Effect of some antibiotics on growth has been reported and their concentration range at which growth inhibition occurs in various cyanobacteria have been listed [1]. Antibiotics like actinomycin-D, puromycin, 5-fluorouracil, chloramphenicol, cyclohexamide, streptomycin, 5-bromouracil and penicillin have been used to study the heterocyst differentiation pattern in an other cyanobacterium, *Anabaena ambigua* Rao [2]. Tyagi [3] reported on the effect of metabolic inhibitors like actinomycin-D, mitomycin-C, DCMU [3-(3,4-dichlorophenyl)-1-dimethyl urea], 2,4-dinitrophenol and sodium azide on heterocyst differentiation of *Anabaena doliolum*. Inhibition of heterocyst development in *Anabaena* by chloramphenicol [4] and streptomycin [5] was reported. Singh [6] also reported inhibition of heterocyst differentiation by chloramphenicol in *Cylindrospermum*. The studies on use of antibiotics in heterocyst differentiation patterns of nitrogen fixing cyanobacteria are mainly concentrated on three species of *Anabaena*, i.e. *A. doliolum* [3], *A. ambigua* [7] and *A. cylindrica* [8]. However, there was no information on the effect of chloramphenicol, rifampicin, puromycin and

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actionmycin-D on heterocyst differentiation pattern of the nitrogen fixing cyanobacterium *Nostoc muscorum*. The present study deals with the effects of these four antibiotics.

Materials and methods

Organism and culture. The axenic culture of nitrogen fixing cyanobacterium *Nostoc muscorum* (Iowa State University strain) was used in the experiment. Its filaments were elongated consisting of an average of 28 ± 2 cells in between two successive spherical heterocysts in nitrogen-free medium and suppression of heterocyst differentiation was noticed in nitrate-containing medium. It was grown in modified Chu-10 medium [9] with trace elements [10] without combined nitrogen source. Cultures were maintained in a culture room at a temperature of 24 ± 2 °C and illuminated with day-light fluorescent tubes for 10 h per day giving an intensity of approximately 3000 lux. Flasks containing 50 ml of cultures were hand shaken daily and were transferred weekly to fresh media to keep the organism in exponential growth phase for using in the experiments.

Antibiotics and growth measurement. The growth experiments were performed in culture tubes (18 × 150 mm) containing 9.5 ml of sterilized combined N-free medium with varying concentrations of chloramphenicol (0, 1.0, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0 µg/ml), rifampicin (0, 0.01, 0.025, 0.05, 0.075, 0.1, 1.0 µg/ml), puromycin and actinomycin-D (0, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 20.0 µg/ml) which were added to the medium after cooling. Aliquots of 0.5 ml of exponentially growing culture were used as inoculum. Growth was measured by Systronic double cell spectrophotometer using red filter of 625–700 nm range. Heterocyst frequency was calculated from the total number of vegetative cells and heterocyst present in filaments of each culture and mean of 30 readings is plotted. Each experiment was conducted in duplicate and repeated twice. The mean values are given in all the figures. Optical density values in the figures on 0 day represent initial OD of culture after inoculation.

Results

Chloramphenicol. The growth of *N. muscorum* in the presence of chloramphenicol declined with increasing the concentration, and at 6.0 µg/ml no growth was noticed (Fig. 1). At levels above 2.0 µg/ml, the culture appeared yellowish and the filaments broke up. Heterocyst frequency was not affected significantly up to 3.0 µg/ml, whereas at 4.0 µg/ml heterocysts could not differentiate (Fig. 2).

Rifampicin. The growth rate of cyanobacterium in the presence of rifampicin declined with increasing the concentration. The growth was almost similar in the untreated control and at 0.0125 µg/ml, but a decline occurred at 0.025 to 0.1 µg/ml and at 1.0 µg/ml growth was inhibited (Fig. 3). At 0.025 µg/ml, the culture became yellowish after 9 days incubation. Heterocyst frequency was not affected significantly even at 0.1 µg/ml (Fig. 4) in liquid medium. On agar plates, filaments with chains of 2–6 heterocysts were noticed in the presence of 0.02 µg/ml, in contrast to the untreated control.

Puromycin. The growth of cyanobacterium in the presence of puromycin declined with the increase of its concentrations (1.0–10.0 µg/ml) and at 8.0 µg/ml, growth was absent (Fig. 5). The culture became yellowish, vacuolated

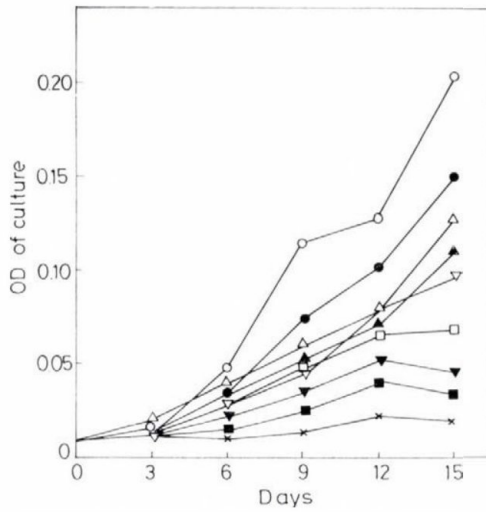


Fig. 1. Growth of *N. muscorum* at different concentrations of chloramphenicol in N-free medium. Control ○—○; 1.0 µg/ml ●—●; 1.5 µg/ml △—△; 2.0 µg/ml ▲—▲; 2.5 µg/ml ▽—▽; 3.0 µg/ml □—□; 4.0 µg/ml ▼—▼; 5.0 µg/ml ■—■; and 6.0 µg/ml ×—×

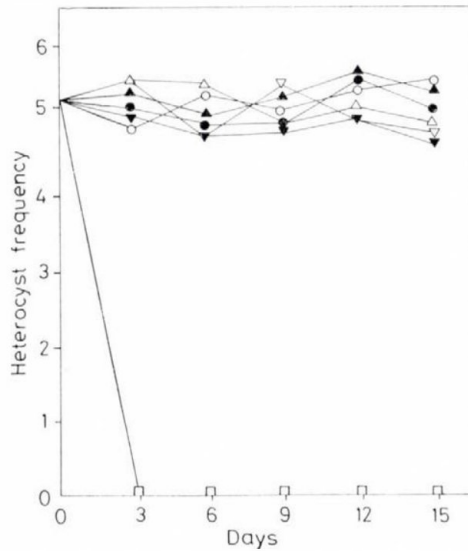


Fig. 2. Heterocyst frequency in *N. muscorum* at different concentrations of chloramphenicol in N-free medium. Control ○—○; 1.0 and 1.5 µg/ml △—△; 2.0 µg/ml ▲—▲; 2.5 µg/ml ▽—▽; 3.0 µg/ml □—□; and 4.0 µg/ml ▼—▼

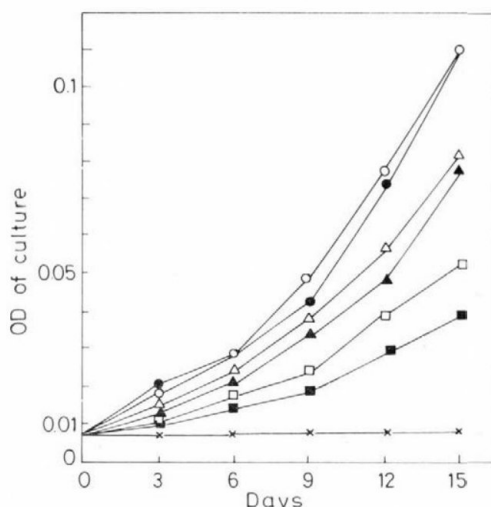


Fig. 3. Growth of *N. muscorum* at different doses of rifampicin in N-free medium. Control ○—○; 0.0125 µg/ml ●—●; 0.025 µg/ml △—△; 0.05 µg/ml ▲—▲; 0.075 µg/ml □—□; 0.1 µg/ml ■—■; and 1.0 µg/ml ×—×

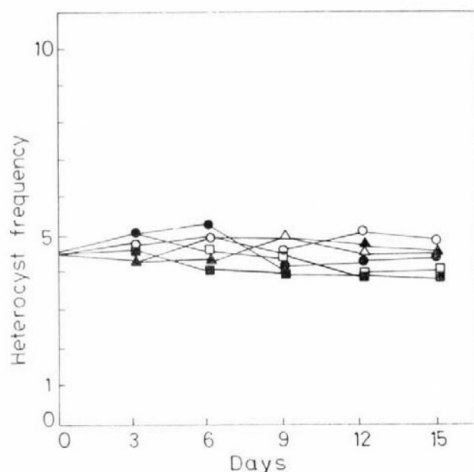


Fig. 4. Heterocyst frequency in *N. muscorum* at different doses of rifampicin in N-free medium. Control ○—○; 0.0125 µg/ml ●—●; 0.025 µg/ml △—△; 0.05 µg/ml ▲—▲; 0.075 µg/ml □—□; and 0.1 µg/ml ■—■

and granulated at higher concentrations. There was a gradual decrease in the heterocyst frequency with increasing the concentrations and 6.0 µg/ml of the drug suppressed heterocyst differentiation after 12 days incubation. Complete suppression of heterocyst differentiation occurred at 8.0 µg/ml (Fig. 6).

Actinomycin-D caused no significant changes in growth rate (Fig. 7) and heterocyst frequency of the organism (Fig. 8) even at 10.0 and 20.0 µg/ml.

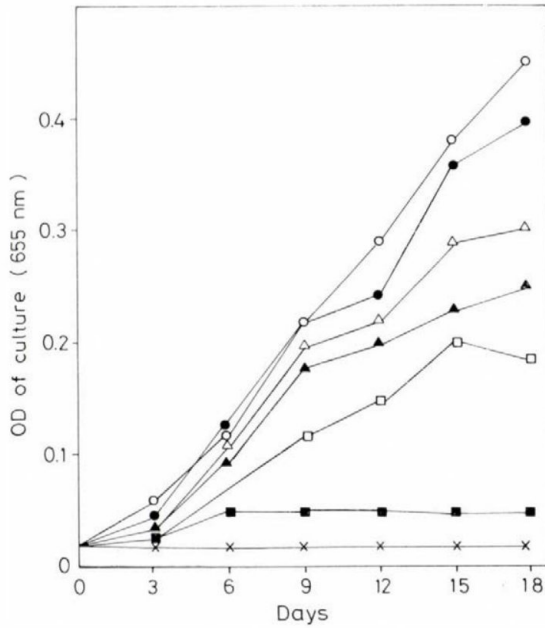


Fig. 5. Growth of *N. muscorum* at different doses of puromycin in N-free medium. Control ○—○; 1.0 µg/ml ●—●; 2.0 µg/ml △—△; 4.0 µg/ml ▲—▲; 6.0 µg/ml □—□; 8.0 µg/ml ■—■; and 10.0 µg/ml ×—×

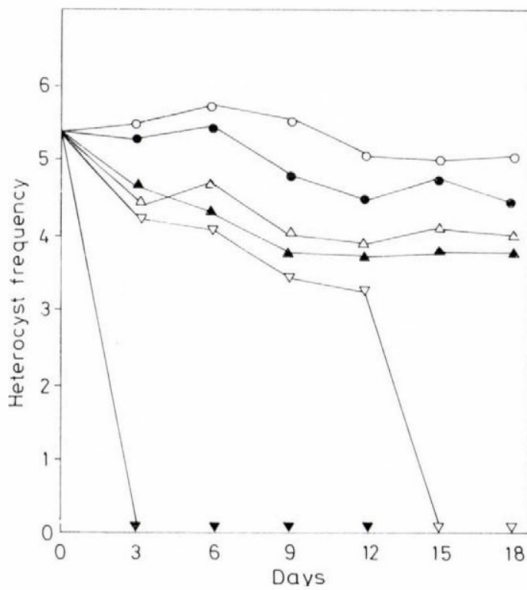


Fig. 6. Heterocyst frequency in *N. muscorum* at different doses of puromycin in N-free medium. Control ○—○; 1.0 µg/ml ●—●; 2.0 µg/ml △—△; 4.0 µg/ml ▲—▲; 6.0 µg/ml ▽—▽; and 8.0 µg/ml ▼—▼

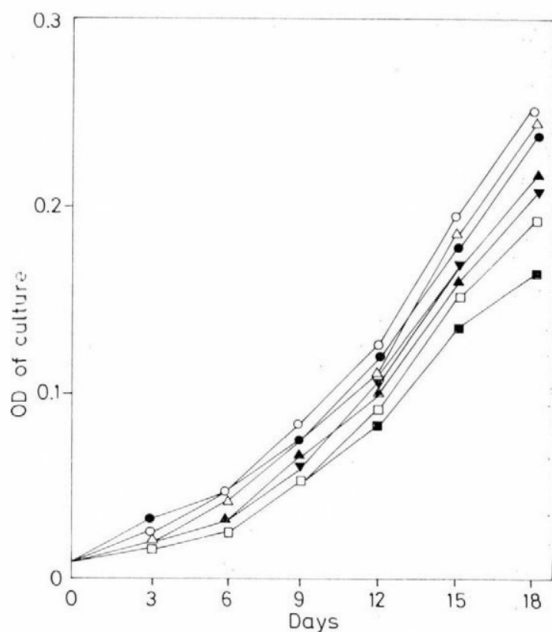


Fig. 7. Growth of *N. muscorum* at different doses of actinomycin-D in N-free medium. Control ○—○; 2.0 µg/ml ●—●; 4.0 µg/ml △—△; 6.0 µg/ml ▲—▲; 8.0 µg/ml ▼—▼; 10.0 µg/ml □—□; and 20.0 µg/ml ■—■

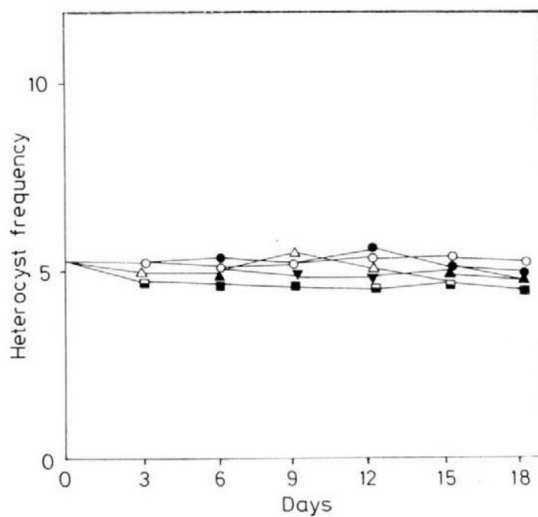


Fig. 8. Heterocyst frequency in *N-muscorum* at different doses of actinomycin-D in N-free medium. Control ○—○; 2.0 µg/ml ●—●; 4.0 µg/ml △—△; 6.0 µg/ml ▲—▲; 8.0 µg/ml ▼—▼; 10.0 µg/ml □—□; and 20.0 µg/ml ■—■

Discussion

Chloramphenicol is known to interfere with protein synthesis by binding to the ribosomes in *Oscillatoria* [11]. Growth inhibition by chloramphenicol at 6.0 $\mu\text{g/ml}$ and inhibition of heterocyst differentiation at 4.0 $\mu\text{g/ml}$ in *N. muscorum* indicated that the heterocyst differentiation process was more sensitive to the antibiotic than the proliferation of vegetative cells. The inhibitory effect on heterocyst differentiation might be due to its effect on proteins (enzymes) involved in cell wall formation since the breakage of the filaments was commonly found. The pigment synthesis also might be impaired since yellowing of culture occurred with increasing the concentration.

Rifampicin acts as an inhibitor in the synthesis of DNA-dependent RNA polymerase. The addition of rifampicin resulted in inhibition of growth in liquid medium and formation of chains of heterocysts in the filaments on solid media. Heterocyst chains on solid media with rifampicin may be associated with the presence of glutamine synthetase (GS) or GOGAT or their products in levels below the critical concentration controlling heterocyst production [12–14]. This may be due to an inhibitory effect of rifampicin on the synthesis of GS and GOGAT. Alternatively, rifampicin may exert a direct inhibitory effect on nitrogenase enzyme synthesis and thus, a consequent reduction in the amount of ammoniacal compounds decrease the activity of GS or GOGAT. Similar results of chain of heterocyst formation in *Anabaena cylindrica* have been reported after rifampicin [8] and azatryptophan treatments [15].

Both puromycin and actinomycin-D are known to inhibit protein synthesis at transcription and translation levels. Puromycin inhibited growth and heterocyst frequency of *N. muscorum*, however, its mode of action still awaits elucidation, since it may inhibit any of the stages of intermediary metabolism.

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SELECTIVE QUANTITATIVE DETERMINATION OF TOBRAMYCIN FROM FERMENTATION BROTH

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A derivative of *Rhizobium meliloti* 41 was constructed (strain GY654) which was resistant to apramycin and kanamycin but remained sensitive to tobramycin. Strain GY654 selectively measures tobramycin and 6''-O-carbamoyltobramycin in the presence of apramycin, kanamycin and 6''-O-carbamoylkanamycin B, therefore it is suitable for the rapid quantitation of 6''-O-carbamoyltobramycin and tobramycin in fermentation broths of *Streptomyces tenebrarius* and solvents containing antibiotic mixtures.

Streptomyces tenebrarius produces a mixture of aminocyclitol antibiotics called nebramycin complex [1] which consists of three main components, apramycin, 6''-O-carbamoylkanamycin B, and 6''-O-carbamoyltobramycin as well as some minor components [2]. Apramycin is used in veterinary practice while alkaline hydrolysis of 6''-O-carbamoylkanamycin B and -tobramycin results in the clinically applied kanamycin B and tobramycin, respectively. Since tobramycin proved to be a valuable tool in the treatment of staphylococcal and pseudomonas infections [3], its industrial production is of great importance. The indicator bacteria used for the microbiological quantitation of antibiotics were sensitive to all components tested, therefore, the diameter of the inhibition zone in agar diffusion test would have been the resultant of the biological activity of the nebramycin complex. Indicator bacteria resistant to apramycin and 6''-O-carbamoylkanamycin B would essentially measure 6''-O-carbamoyltobramycin in a simple agar diffusion test.

In this paper we describe the construction of an apramycin and 6''-O-carbamoylkanamycin resistant derivative of *Rhizobium meliloti* 41 and demonstrate that this strain is capable of the exclusive quantitation of 6''-O-carbamoyltobramycin in fermentation broths.

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Materials and methods

Strains. The microorganisms, plasmid and transposon used in this study were: *Rhizobium meliloti* 41 derivatives AK 631, GY625 and GY654; *Staphylococcus epidermidis* ATCC 12228; *Bacillus subtilis* ATCC 6633; *Streptomyces tenebrarius*; *Escherichia coli* J53, plasmid pJB4JI and transposon Tn5 [4, 5].

Media. Complete GTA and peptone-casein-agar (PCA) media were described by Kiss et al. [6] and Oden et al. [7], respectively. For measuring the antibiotic concentration, GPLY medium was used containing 10 g glucose, 5 g peptone, 1.5 g Lab Lemco powder, 1.5 g yeast extract and 15 g Bacto agar. The ingredients were dissolved in 1000 ml distilled water, and the pH was adjusted to 7.5 before sterilization.

Mating was performed on agar surface as described before [8, 9], except that the modified media of Kiss et al. [6] were used.

Agar diffusion test. Large plate agar diffusion technique was done as described [10]. Aliquots of 250 ml GPLY agar medium were inoculated with the indicator strain and poured onto a glass plate. Ten mm holes were cut out from the agar and 0.1 ml of solution containing appropriate antibiotic standards or fermentation broths were pipetted into the holes. After 20 h incubation at 37 °C the diameters of the inhibition zones were measured. Bioautographic detection and quantitation of antibiotics was performed as described by Kádár—Pauncz and Harsányi [11].

Antibiotics. Standard antibiotic solutions were prepared in 10 mM phosphate buffer, pH 7.5. Tobramycin base was obtained from Biogal Pharmaceutical Factory (Debrecen, Hungary). Apramycin, kanamycin B, 6''-O-carbamoylkanamycin B and 6''-O-carbamoyltobramycin were isolated from the broth of *S. tenebrarius*.

Results

It has previously been found that *R. meliloti* 41 was sensitive to 6''-O-carbamoyltobramycin, apramycin and 6''-O-carbamoylkanamycin B, therefore, using agar diffusion test, the strain would measure the total biological activity of all antibiotics present. To circumvent the apramycin and kanamycin B sensitivity, resistant derivatives of *R. meliloti* 41 were isolated. Kanamycin B resistant derivative was isolated by introducing Tn5 transposon into *R. meliloti* 41 carrying kanamycin resistant determinant [5]. This was conducted in mating experiment by conjugation of *E. coli* J53 (*pro*, *met*) carrying plasmid pJB4JI (Gm, Sm::Mu::Tn5, *incPl*), see Beringer et al. [4] with derivative of *R. meliloti* 41 (streptomycin resistant, compact colony morphology, strain AK684). Kanamycin resistant *R. meliloti* 41 transconjugants appeared at a frequency of 5×10^{-6} due to the transposition of Tn5 into the genom of the recipient [4, 12]. One Km^R colony was purified twice for single colonies and denoted GY625. Strain GY625 failed to give inhibition zone in agar diffusion test in the presence of 3200 µg/ml kanamycin B but retained its original sensitivity to apramycin and tobramycin (data not shown). Apramycin resistant derivative was isolated spontaneously by plating 5×10^{10} bacteria GY625 onto GTA plate containing 800 µg/ml apramycin. After five days of incubation at 34 °C, one of these resistant colonies was purified and denoted GY654.

R. meliloti 41 strain GY654 was used as an indicator microorganism in agar diffusion test to check its sensitivity toward the major factors of the nebramycin complex. Twenty mm inhibition zone was produced by 6600 µg/ml

apramycin, 6000 $\mu\text{g/ml}$ 6''-O-carbamoylkanamycin B and 96 $\mu\text{g/ml}$ 6''-O-carbamoyltobramycin. The linear calibration curve for 6''-O-carbamoyltobramycin (logarithm of the concentration of the antibiotic versus the diameter of the inhibition zone) was between 12.5–100 $\mu\text{g/ml}$.

The large degree of differences in sensitivity of GY654 towards the antibiotics and the linear interval of the calibration curve in the bioassay made possible the exclusive detection and quantitation of 6''-O-carbamoyltobra-

Table I

Quantitation of 6''-O-carbamoyltobramycin from fermentation broths

No. of sample	Concentration ($\mu\text{g/ml}$) measured by bioautography			Biological activity ($\mu\text{g/ml}$)	
	Apra-mycin	6''-O-carbamoylkanamycin B	6''-O-carbamoyltobramycin	Indicator strain	
				GY654	<i>S. epidermidis</i> ATCC 12228
1	1400	532	863	797	1190
2	2300	0	1154	1057	1385
3	1480	720	851	802	1245
4	0	1600	1500	1505	2850
5	3200	560	1480	1510	2420

mycin in the presence of apramycin and 6''-O-carbamoylkanamycin B (see Table I). The biological activity of five independent fermentation samples was determined by different methods. The first three column demonstrates the concentration of the three major fermentation end products deduced from the quantitative bioautography after thin layer chromatography [11]. In these cases the proper standard solution for each antibiotic was used for calibration curves and *B. subtilis* ATCC 6633 as indicator organism. The fourth and the fifth column shows the biological activity of the fermentation broths determined by the large plate agar diffusion technique using *R. meliloti* strain GY654 and *S. epidermidis* ATCC 12228, respectively. It can be seen that GY654 measures the actual concentration of 6''-O-carbamoyltobramycin in the fermentation broths.

Discussion

In this paper we described the construction of an apramycin and kanamycin B resistant derivative of *R. meliloti* 41. This strain GY654 is sensitive to 6''-O-carbamoyltobramycin but resistant to the two other major compo-

nents of the nebramycin complex produced by *S. tenebrarius*. This phenotype enables strain GY654 to measure the actual concentration of 6''-O-carbamoyltobramycin.

The strain construction was started from a spontaneously isolated streptomycin resistant derivative of *R. meliloti* 41 (strain AK684). The mutation is probably located on the chromosome of *R. meliloti* 41 [9] and altered the small ribosomal protein *rpsL* [13]. For providing kanamycin B resistance to this strain, we introduced transposon Tn5. Tn5 codes for two aminoglycoside antibiotic resistant determinants [12, 14]. The streptomycin resistant gene probably inactivates streptomycin but not tobramycin [14], therefore its presence can be negligible. The kanamycin resistant gene codes for an enzyme called aminoglycoside phosphotransferase (3') type II (APH(3')II). This enzyme inactivates kanamycin B by phosphorylating the 3' OH-group [15]. This 3' C-atom carries an H-group in tobramycin, therefore is not a substrate of APH(3')II. The Tn5 derivative of *R. meliloti* 41, strain GY625 confers resistance to kanamycin B but not to tobramycin. The apramycin resistant character was produced spontaneously. The nature of this mutation is unknown.

R. meliloti 41, strain GY654 is a very useful analytical tool to measure selectively the concentration of 6''-O-carbamoyltobramycin in fermentation broths containing nebramycin antibiotic complex. This method has been used routinely for quantitation of 6''-O-carbamoyltobramycin in large scale fermentations and strain screening experiments since years.

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COMPARATIVE EFFECTS OF SYNTHETIC INSECTICIDES — ENDOSULFAN, PHOSALONE AND PERMETHRIN — ON *CHLAMYDOMONAS* *REINHARDTII* ALGAL CELLS

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Presence of insecticide-endosulfan, phosalone or permethrin in the growth medium caused concentration dependent inhibition in the vegetative growth of *Chlamydomonas reinhardtii* algal cells. The rate of inhibition produced by endosulfan was two-fold higher than that of phosalone or permethrin. Endosulfan affected the cell growth completely at 100 times less concentration as compared to that of phosalone or permethrin. Non-dividing cell populations encountered significant losses in cells during their exposure (2 h) to endosulfan and did not show further loss in the 72 h post-treatment period. The populations treated with phosalone exhibited losses of considerable magnitude in the post-treatment period. Permethrin treated non-dividing cell populations did not lower the cell number, either after the treatment period (2 h) or the post-treatment period (72 h). However, these populations showed reduced levels of chlorophyll following the exposure of the insecticide and did not display recovery or further reduction in the levels in the post-treatment period. The chlorophyll levels of endosulfan or phosalone treated cell populations remained unaffected. The cells remaining intact after the treatment (2 h) of endosulfan or phosalone exhibited significant decreases in their post treatment vegetative growth abilities. The growth ability of such permethrin exposed cells was similar to that of untreated cells.

Biological activities of insecticides do not only remain confined to target organisms but also extend to non-target organisms which play an important role in the maintenance of harmony in the eco-system. Algal cells constitute a major portion of the non-target organisms and thus, comprehension of the action of insecticides on these cells is of importance [1–3]. Algal cells of *Chlamydomonas reinhardtii* offer a suitable model system for such ecotoxicological studies as this organism can be easily grown, asexually and sexually, in laboratory [4, 5].

This paper reports on comparative effects of synthetic insecticides — endosulfan, phosalone and permethrin — belonging to different chemical classes (organochlorine, organophosphorous and pyrethroid, respectively).

Materials and methods

Cell culture. Cell culture of *C. reinhardtii* WT (gifted by Dr. P. E. Brayant, Institute for Biology, Frankfurt, Main, FRG) was grown in synthetic medium under continuous light (3750 lux) from a band of fluorescent tubes for 72 h at 25 °C [6].

Synthetic insecticides. The purity of technical grade endosulfan (Excel Industries, Bombay, India), phosalone (Volrho Ltd., Hyderabad, India) and permethrin (The Alkali Chemical Corporation, Ltd., Calcutta, India) was 94, 97 and 93%, respectively. The concentration of each insecticide expressed in the experiments is based upon the purity of the respective chemical. Solutions of endosulfan and phosalone were prepared in dimethyl sulfoxide (DMSO, A. R. Grade, Sigma Chemical Company, USA). The solution of permethrin was made in absolute ethyl alcohol. Solutions of the insecticides were sterilized by filtration through fluopore filters (pore size 45 µm) and diluted to the desired concentrations with presterilized growth medium. In all experiments, the concentration of the organic solvents (DMSO and ethyl alcohol) in the growth medium was maintained at or less than 2%. DMSO or ethyl alcohol at the concentration of 2% in the medium did not affect the vegetative growth of *C. reinhardtii* cells.

Cell count. *C. reinhardtii* cells after fixation in formaldehyde solution (5 per cent, v/v) were counted by the use of haemocytometer. The cell count of each sample was taken in triplicate.

Estimation of total chlorophyll. Cells (1×10^7) from the growth medium were collected by centrifugation, washed twice with distilled water and chlorophyll was extracted with acetone : water (80 : 20, v/v; 5 ml) at 25 °C [7]. The solvent containing chlorophyll was separated by centrifugation and used for the estimation of the pigment [8].

Non-dividing cell-suspension. Freshly grown cells were collected by centrifugation, washed with the growth medium, suspended in the same medium at three different cell densities (5×10^5 , 5×10^6 and 5×10^7 cells/ml), and incubated for 72 h at 25 °C in continuous light. These experimental steps were performed under sterile conditions. At 24, 48 and 72 h of the incubation periods, aliquots were drawn, cells collected by centrifugation, washed twice with distilled water and suspended for determination of cell number, total protein and chlorophyll contents as described above. Cells in the 5×10^6 cells/ml suspension, did not show any change in cell number, total protein and chlorophyll contents up to 72 h. Cells in the 5×10^5 suspension showed vegetative growth (about 20% increase over the original cell number) and those in the 5×10^7 suspension exhibited decrease in total protein content (12%) and total chlorophyll content (8%) (results not included). Suspension 5×10^6 cells/ml, termed as "non-dividing cell suspension", was chosen for experiments on non-dividing cells.

Vegetative growth in the presence of endosulfan, phosalone and permethrin. Growth medium (20 ml) containing different concentrations of endosulfan (10^{-6} M– 10^{-5} M; 0.407–4.07 ppm), phosalone (10^{-5} M– 10^{-3} M; 3.68–368 ppm) and permethrin (10^{-5} M– 10^{-3} M; 3.91–391 ppm) in Erlenmeyer flasks (100 ml capacity) was inoculated with 0.5 ml of 72 h grown culture ($\approx 2.5 \times 10^6$ cells), incubated at 25 °C for 72 h in continuous light and vegetative growth was measured by cell count.

Effects of endosulfan, phosalone and permethrin treatment on non-dividing cell-population. The suspension of non-dividing cells (100 ml, 5×10^6 cells/ml of growth medium) was incubated at 25 °C for 72 h in continuous light after addition of endosulfan (2.5×10^{-5} M, 10.2 ppm), phosalone (1×10^{-3} M, 368 ppm) or permethrin (1×10^{-3} M, 391 ppm). At the desired incubation periods (2, 4, 6, 8, 16, 24, 48 and 72 h), aliquots were drawn for determination of cell number and chlorophyll content as described above.

Post-treatment vegetative growth of endosulfan phosalone and permethrin treated cells. Cells in non-dividing suspension condition (20 ml, 5×10^6 cells/ml) were incubated at 25 °C for 72 h in continuous light in the presence of endosulfan (2.5×10^{-5} M, 10.2 ppm), phosalone (1×10^{-3} M, 368 ppm) or permethrin (1×10^{-3} M, 391 ppm). At different incubation periods, aliquots (2 ml) were withdrawn, cells collected by centrifugation, washed twice with sterilized growth medium and resuspended in the same medium (5×10^6 cells/ml). To assess the vegetative growth ability of the insecticide treated cells, 0.5 ml of the suspension was inoculated in 20 ml medium and incubated at 25 °C for 72 h in continuous light. At 24, 48 and 72 h of the incubation, aliquots (2 ml) were drawn for the measurement of vegetative growth by cell count.

Results and discussion

The vegetative growth response (measured at 72 h of the growth period) of *C. reinhardtii* WT cells in the presence of different concentrations of insecticide — endosulfan, phosalone or permethrin — is shown in Fig. 1. It is discernible that endosulfan, phosalone and permethrin, at the concentration of 1.0×10^{-6} M (0.4 ppm), 1.2×10^{-5} M (4.4 ppm) and 1.2×10^{-5} M (4.7 ppm), respectively, do not affect the vegetative growth, but at the respective con-

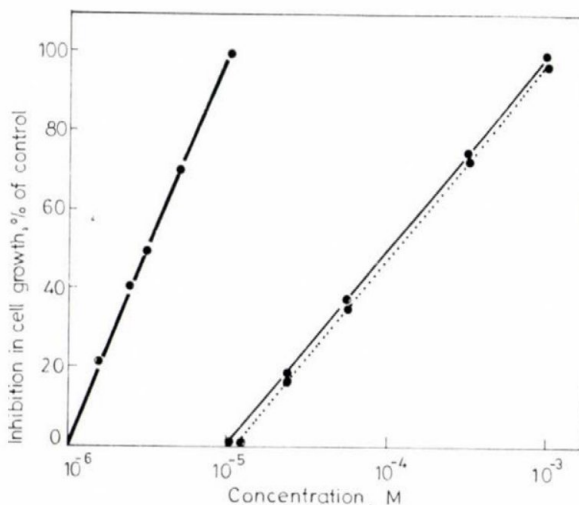


Fig. 1. Vegetative growth of *C. reinhardtii* WT cells in the presence of endosulfan, phosalone or permethrin. Freshly grown cells (10^6 cells/ml, 0.5 ml) were inoculated in growth medium (20 ml) containing different concentrations of the insecticide, incubated at 25 °C for 72 h in light and the vegetative growth was measured by cell count. Each point represents a mean of four replicates of five independent experiments. Endosulfan, ●—●; phosalone, ●- - -●; permethrin, ●.....●

centrations of 1×10^{-5} M (4.0 ppm), 1×10^{-3} M (368.0 ppm) and 1×10^{-3} M (391.0 ppm), inhibit the same completely. Endosulfan, phosalone and permethrin induce dose dependent growth inhibition in the concentration range between non-toxic dose (a dose that does not affect vegetative cell growth) and toxic dose (a dose that inhibits vegetative growth completely), the respective ranges being 1×10^{-6} M to 1×10^{-5} M (0.4 to 4.0 ppm), 1.2×10^{-5} M to 1×10^{-3} M (4.4 to 368.0 ppm) and 1.2×10^{-5} M to 1.0×10^{-3} M (4.7 to 391.0 ppm). The toxic dose (1×10^{-6} M) of endosulfan is about 100 times lower than those of phosalone (1×10^{-3} M) and permethrin (1×10^{-3} M). The slopes of growth inhibition plots of endosulfan, phosalone and permethrin work out to be 2.6, 1.3 and 1.3, respectively. The slopes indicate that the rate of inhibition in the growth caused by endosulfan is two fold higher than those of phosalone and permethrin.

Effects of the insecticide treatment (at toxic dose level; endosulfan, 2.5×10^{-5} M; phosalone, 1.0×10^{-3} M; permethrin, 1.0×10^{-3} M) for different periods (2, 4, 6, 8, 16, 24, 48 and 72 h) on non-dividing *C. reinhardtii* cell population are shown in Fig. 2. Treatments with endosulfan for 2 and 8 h induced loss in the cell population of about 45 and 100%, respectively. The loss increased with the increase in the treatment period from 2 to 8 h. Phosalone treatment from 2 to 24 h also exhibited such effect on the cell population,

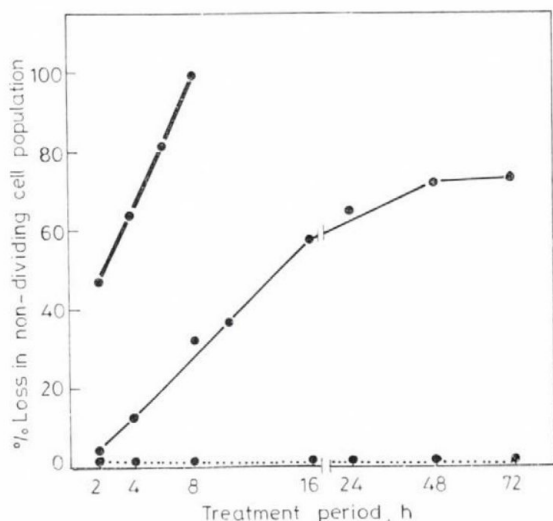


Fig. 2. Effects of endosulfan, phosalone or permethrin treatment on non-dividing cell population of *C. reinhardtii* WT. Cells in non-dividing suspension condition containing endosulfan (2.5×10^{-5} M), phosalone (1×10^{-3} M) or permethrin (1×10^{-3} M) were incubated at 25 °C for 72 h in light and aliquots were drawn at different periods for determination of cell count. Each point represents a mean of four replicates of five independent experiments. Endosulfan, ●—●; phosalone, ●—●; permethrin, ●.....●

the loss of cell population rising from 4 to 65% with the increase in the treatment period. The marginal increase in the loss (from 65 to 75%) with the extension of the treatment period from 24 to 72 h could be due to development of phosalone resistance in the cells in the early phase (24 h) of the treatment [9]. Endosulfan and phosalone may induce losses in non-dividing cell populations by inflicting damages in cell-membrane [10–12] and/or by releasing hydrolytic enzymes from vacuoles of the cells [13, 14]. Unlike endosulfan and phosalone, permethrin does not induce loss in non-dividing cell population, even after a treatment of 72 h.

Results on the post-treatment vegetative growth abilities at 24, 48 and 72 h periods of endosulfan (2.5×10^{-5} M), phosalone (1.0×10^{-3} M) and permethrin (1.0×10^{-3} M) treated (in non-dividing condition) cells are shown in Fig. 3. Cells treated with endosulfan for 2, 4 and 6 h (Fig. 3a) exhibited

about 60% reduction in vegetative growth in the first 24 h post-treatment period and did not show any change in the next 48 h of the growth period (from 24 to 72 h). Additionally, the level of reduction did not alter with the increase in endosulfan treatment period (from 2 to 6 h). The growth ability of cells treated with endosulfan for 8 h could not be examined, as all of the cells became lysed during this period.

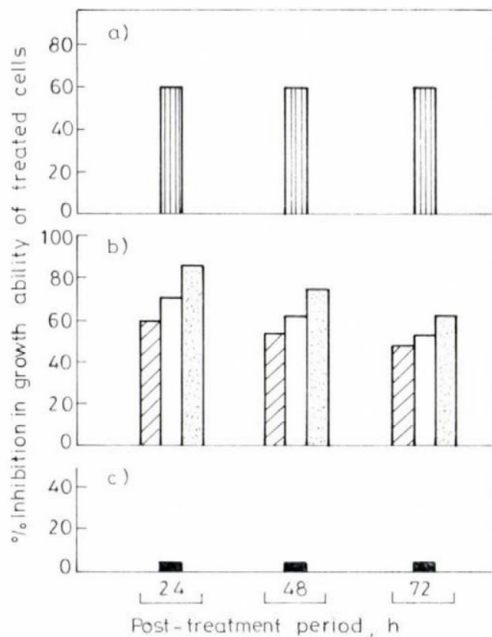


Fig. 3. Vegetative growth of endosulfan, phosalone or permethrin treated *C. reinhardtii* WT cells. Cells in non-dividing condition (5×10^6 cells/ml) were treated with endosulfan (2.5×10^{-5} M), phosalone (1×10^{-3} M) or permethrin (1×10^{-3} M) at 25°C in light for 72 h. At different time intervals of the treatment (2 to 72 h), aliquots were drawn, cells collected, washed and resuspended in fresh growth medium (5×10^6 cells/ml). The cells (5×10^6 cells/ml, 0.5 ml) were inoculated in fresh growth medium (20 ml) and incubated at 25°C in continuous light for 72 h. At 24, 48 and 72 h of the growth period, aliquots were drawn for determination of cell count. Each value represents a mean of four replicates of five independent experiments. Significance, $P < 0.05$. (a) Cells treated with endosulfan for 2, 4, 6 h. (shaded columns); (b) Cells treated with phosalone for 2, 4, 8, 24 h (hatched columns); for 48 h (open columns); for 72 h (stippled columns); (c) Cells treated with permethrin for 2, 4, 8, 24, 48, 72 h (solid columns)

Cells treated with phosalone (Fig. 3b) for 2–24 h, 48 h and 72 h exhibited, respectively, 60, 71 and 85% inhibition in vegetative growth at 24 h post-treatment period; at 48 and 72 h of this period, these levels lowered to 55, 61, 76% and 48, 53, 62%, respectively. The lowering in the inhibition of vegetative growth indicate mild recovery in phosalone treated cells. Examination of the post-treatment vegetative growth inhibition patterns of endosulfan or phosalone treated cells (Fig. 3a, 3b) would point out that the quantum

of cell injury produced in 2 h exposure to these insecticides can cause drastic inhibition (more than 50%) in vegetative cell growth.

Cells treated with permethrin (Fig. 3c) up to 72 h show marginal reduction (3–4%) in post-treatment growth ability. The effect of permethrin on post-exposure vegetative cell growth is strikingly less than that of endosulfan or phosalone.

Results on the effects of endosulfan (2.5×10^{-5} M), phosalone (1×10^{-3} M) permethrin (1×10^{-3} M) treatment (2 h) on non-dividing cell population and

Table I

Effect of insecticides on cell number and chlorophyll content of non-dividing C. reinhardtii cells

Insecticide	Post-treatment period (h) in non-dividing cell condition			
	0	24	48	72
Endosulfan				
% cell loss	45.0 ± 1.6	47.6 ± 0.9	46.0 ± 1.7	44.0 ± 1.2
% decrease in chlorophyll	nil	nil	nil	nil
Phosalone				
% cell loss	4.0 ± 0.6	52.0 ± 1.1	49.6 ± 2.7	51.7 ± 0.7
% decrease in chlorophyll	nil	nil	nil	nil
Permethrin				
% cell loss	1.5 ± 0.03	1.6 ± 0.02	2.4 ± 0.05	2.1 ± 0.02
% decrease in chlorophyll	8.5 ± 1.1	8.2 ± 0.6	7.6 ± 0.3	8.0 ± 0.5

Cells in non-dividing suspension condition were treated for 2 h with endosulfan (2.5×10^{-5} M), phosalone (1×10^{-3} M) or permethrin (1×10^{-3} M), collected by centrifugation, washed, resuspended in medium (5×10^6 cells/ml) as per non-dividing cell condition and kept in light for 72 h at 25 °C. At 0, 24, 48, 72 h of the post-treatment period, aliquots were drawn for the measurement of cell number and total chlorophyll content. The cell number and chlorophyll content of untreated cells in non-dividing condition did not change in the 72 h period. Each value represents a mean of four replicates of five independent experiments $\pm$ SEM.

cellular chlorophyll content at 24, 48 and 72 h post-treatment periods are given in Table I. The significant loss in non-dividing cell population in the 2 h treatment period of endosulfan and none in the 72 h post-treatment period indicate that the loss in population occurs when the cells are in contact with endosulfan. Such loss in the cell population may take place due to a heavy leakage of cell-cytoplasm through endosulfan-induced cell wall injury [10, 15]. The magnitude of such injury in the cells which remain intact following endosulfan treatment, may not be sufficient enough to cause the leakage of cytoplasm. On the other hand, phosalone treated non-dividing cell population, which manifest a marginal cell loss during the treatment period (2 h), exhibit a loss of high magnitude (around 50%) in the first 24 h post-treatment period and in the next 48 h post-treatment period (24 to 72 h), did not show additional loss in cells (Table I). Such loss in cells may occur due

to excessive leakage of cytoplasm through cell wall lesions [10] produced during the treatment period (2 h) and enlarged in the early phase of post-treatment periods (2 h) and/or due to lysing through the action of hydrolysing enzymes leaked by way of lesions produced in vacuolar membrane [13, 14].

As shown in Table I, endosulfan and phosalone, which induce significant losses in non-dividing cell populations, did not affect cellular chlorophyll levels, either during the treatment period (2 h) or during the post-treatment period (72 h). On the other hand, permethrin, which does not cause loss in non-dividing cell population, either in the treatment or in the post-treatment periods, reduced the chlorophyll content of cells during the treatment period (2 h). The lowered content of chlorophyll did not alter in the 72 h post-treatment period. Further work on this observation would yield interesting information.

Results from this paper indicate that endosulfan, phosalone and permethrin belonging to three different chemical classes — organochlorine, organophosphorous and pyrethroid, respectively — induce deleterious effects of different degrees on dividing and non-dividing algal cells of *C. reinhardtii*. The intensities of such effects induced by endosulfan and phosalone are much stronger than that by permethrin.

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UTILIZATION OF ORGANIC SUBSTRATES BY TWO FILAMENTOUS CYANOBACTERIA UNDER VARIOUS GROWTH CONDITIONS

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Two filamentous cyanobacteria *Calothrix marchica* Lemm. var. *intermedia* Rao and *Scytonema schmidlei* De Toni grew heterotrophically in light with or without DCMU and in the dark when supplemented with glucose, fructose or sucrose. Galactose, mannitol and sorbitol were utilized less efficiently. Mannose, xylose and organic acids did not support the heterotrophic growth of both the cyanobacteria. By continuous incubation and sub-cultivation in sucrose supplemented medium in the dark, heterotrophic adapted strains of the organisms were produced. These strains, in contrast to the light pre-treated ones, readily utilized the exogenous substrate as evidenced by increase in growth and the total nitrogen content of the cultures.

In continuation of the investigation on the heterotrophic growth characteristics of various cyanobacteria [1–5] the present work was undertaken to study the effect of various exogenous carbon compounds on the growth characteristics of two filamentous cyanobacteria *Calothrix marchica* Lemm. var. *intermedia* Rao and *Scytonema schmidlei* De Toni, isolated from a distillery effluent polluted habitat [6]. Since these cyanobacteria occurred abundantly in the soil of the polluted fields rich with reducing sugars of the distillery waste [7], it was thought that they must be utilizing the exogenous organic matters for growth and development.

Materials and methods

The cyanobacteria *C. marchica* (strain Sahu 1978/3) and *S. schmidlei* (strain Sahu 1978/4) [6] were grown in Allen and Arnon's nitrogen-free medium [8] at $24 \pm 2^\circ\text{C}$ under white fluorescent tubes at an intensity of 2200 lux. The effect of various concentrations of a wide range of carbon compounds comprising sugars, sugar alcohols and organic acids on the growth of the organisms were studied in light in presence or absence of 10^{-5} M DCMU (the concentration at which the growth of both the cyanobacteria were completely inhibited in the mineral medium in light) and in the dark. The exogenous carbon sources were filter sterilized and added aseptically to the inorganic culture medium to obtain the required concentration of each of the substrate as per the design of the experiment. The growth medium was always adjusted to pH 7.5. Experiments were conducted by inoculation of equal amounts of exponentially growing material (equivalent to 1.0 mg dry weight each) into 25 ml of culture solution. Heterotrophic

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bacterial contamination was monitored by plating the suspension of each of the cyanobacterium from culture vessels on Difco nutrient agar supplemented with glucose. The procedure for determination of growth was described earlier [6]. Total nitrogen content of certain cultures were estimated by nesslerization method [9]. The amounts of nitrogen fixed by different cyanobacterial strains were determined after subtracting the nitrogen present in the initial inoculum.

Results and discussion

The results on the exogenous substrate induced growth of the cyanobacteria *C. marchica* and *S. schmidlei* in the light in the presence or absence of 10^{-5} M DCMU and in the dark are shown in Table I. Both the light pre-treated organisms when grown in the fresh culture medium showed an increase in growth (dry weight basis) in the light. Their growth rates were significantly enhanced when the culture medium was provided with 15–25 mM of fructose,

Table I

Effect of organic substrates on the growth (dry weight in mg) of C. marchica and S. schmidlei under different growth conditions

Mineral medium with	Concentration of substrate (mM)	<i>C. marchica</i>			<i>S. schmidlei</i>		
		Light	Light + 10^{-5} M DCMU	Dark	Light	Light + 10^{-5} M DCMU	Dark
No addition (control)		17.24	2.8	2.4	12.9	2.25	1.67
Sucrose	5	17.92	6.54	4.52	24.04	6.5	5.63
	7.5	18.66	7.82	6.24	29.5	10.3	7.35
	15	21.0	9.95	7.4	38.7	11.8	9.0
	25	19.83	10.0	7.42	38.02	11.95	9.81
	50	18.45	10.2	7.13	37.31	9.4	9.92
Fructose	5	18.49	4.85	3.59	21.0	3.9	2.59
	15	22.5	7.02	6.9	27.4	5.0	3.0
	25	19.82	7.18	7.29	22.4	6.6	4.68
	50	19.49	6.46	7.09	21.0	6.05	4.62
Glucose	5	17.82	4.5	2.89	35.0	4.2	3.29
	15	18.99	6.95	6.66	38.96	5.55	4.94
	25	18.48	6.84	4.65	37.2	6.42	5.99
	50	17.71	6.7	3.39	37.08	6.11	5.87
Galactose	15	17.56	4.55	3.94	12.94	3.86	2.34
Mannose	15	4.0	2.15	2.8	9.48	2.05	2.4
Xylose	15	6.99	2.0	2.4	8.65	1.8	1.75
Mannitol	15	18.21	3.25	2.79	15.4	4.52	2.28
Sorbitol	15	17.84	4.0	2.65	14.11	3.89	2.59
Sodium acetate	15	13.2	2.7	2.4	12.85	2.03	1.85
Fructose 1,6-diphosphate	15	10.35	2.05	2.22	8.65	2.14	1.73
Pyruvic acid	15	4.88	2.0	2.4	4.77	2.08	2.11
Succinic acid	15	8.42	2.0	2.8	4.85	2.0	1.67

Initial inoculum of the cyanobacteria (equivalent to 1.0 mg dry weight each) were cultured in 25 ml of culture medium up to 15 days at 24 ± 2 °C. Each value represent the mean of five closely concordant determinations

glucose or sucrose; very little growth was observed with galactose, mannitol and sorbitol. Glucose and fructose were found to be most efficient to increase the growth of *S. schmidlei* and *C. marchica* respectively in the light. Photoheterotrophic growth did not occur with mannose, xylose, acetate, fructose 1,6-diphosphate, pyruvic acid and succinic acid. In the light (in the presence of 10^{-5} M DCMU) and in the dark, the organisms failed to grow when the medium was not provided with any exogenous carbon compound. However, when an appropriate exogenous substrate was added to the culture medium, it could considerably increase the growth (Table I).

Chemoheterotrophic growth was studied in the light (in the presence of 10^{-5} M DCMU) and in the dark. The objective of the former experimental condition was to block the assimilation of CO_2 indirectly with DCMU which inhibits photosystem II and the production of NADPH. In both the cases only organic carbon compounds provide the source of carbon and energy necessary for the growth and development. Chemoheterotrophic growth was observed in sucrose, glucose and fructose supplemented cultures; sucrose was more efficiently utilized than any other substrate in the dark. Galactose, mannose, xylose, the sugar alcohols, acetate and the organic acids were less efficient to support the growth of both the cyanobacteria under similar experimental conditions. The results also showed that photoheterotrophic and chemoheterotrophic growth of both the experimental organisms were maximum at 15–25 mM concentrations of various substrates; fructose, glucose and sucrose at 15 mM concentrations were more effective than any other substrate tested to increase the growth in the light as well as in the dark. Since glucose and fructose contain half as many carbon atoms as sucrose, if the growth on these compounds is compared on a per C basis, the superiority of fructose and glucose for supporting growth of *C. marchica* and *S. schmidlei* respectively is even more striking (Table I).

These findings tallie with the earlier reports about *Chlorogloea fritschii* [10], *Tolypothrix tenuis* [11], *Anabaena* sp. [4], *Westiellopsis prolifica* [5] etc. for which mostly glucose, fructose or sucrose were the best substrates for heterotrophic growth. However, these reports, together with the earlier findings on various other cyanobacteria [12–14] confirm that the ability of a particular substrate to support heterotrophic growth varies with genera species and strains. Lack of versatility in the use of organic substrates appears to be a result of permeability restrictions [15–18]. The failure to grow on certain organic compounds which are probably intermediates or products of metabolic cycles can be explained by their inability to penetrate the cell membrane for assimilation [19–20]. Though certain exogenous substrates supported the chemoheterotrophic growth, the substrate induced dark growth obtained was only a fraction of the autotrophic growth of both the cyanobacteria. The photostimulation and assimilation of organic carbon compounds

Table II

Effect of sucrose (15 mM) on the light pre-treated, dark pre-treated and heterotrophic adapted strains of *C. marchica* and *S. schmidlei* in relation to growth and nitrogen fixation in light and dark

Medium	Continuous light				Continuous dark			
	<i>C. marchica</i>		<i>S. schmidlei</i>		<i>C. marchica</i>		<i>S. schmidlei</i>	
	Growth dry wt. (mg)	Total nitrogen fixed (mg)/25 ml culture	Growth dry wt. (mg)	Total nitrogen fixed (mg) 25 ml culture	Growth dry wt. (mg)	Total nitrogen fixed (mg)/25 ml culture	Growth dry wt. (mg)	Total nitrogen fixed (mg)/25 ml culture
(i) Light pre-treated (previously grown in the basal inorganic medium in light for 15 days) strain								
Control	17.24	2.52	12.9	1.81	2.5	0.4	1.72	0.3
Sucrose	21.0	3.36	38.7	5.23	9.45	1.51	15.05	2.21
(ii) Dark pre-treated (previously grown in the basal inorganic medium in dark for 30 days) strain								
Control	19.87	2.08	14.27	1.93	1.6	0.27	1.34	0.17
Sucrose	24.75	3.29	42.32	4.75	9.08	1.27	15.22	2.01
(iii) Heterotrophic adapted (previously incubated and sub-cultured in sucrose supplemented medium in dark at 30 days interval up to 90 days) strain								
Control	17.39	2.85	13.68	1.82	2.83	0.53	2.18	0.36
Sucrose	22.8	5.2	40.9	5.92	11.6	2.38	18.7	2.62

Initial inocula of the strains of cyanobacteria (equivalent to 1.0 mg dry weight each) were cultured in 25 ml of medium at $24 \pm 2^\circ\text{C}$. Harvested after 15 days and 30 days of light and dark incubation, respectively. Each value is the mean of five closely concordant determinations

may be connected with the supply of ATP through photophosphorylation which could support energy requiring transport of the exogenous substrates and their subsequent assimilation in the cells. But in the dark, the experimental organisms could absorb and assimilate a small amount of the substrates initially, that could increase the rate of respiration resulting in the production of ATP, which could subsequently be utilized by rapid uptake and assimilation of the substrates. Obviously the process would be slower in the dark than in the light where ATP is readily available by photosynthesis.

By continuous incubation and repeated sub-cultivation in the dark with 15 mM sucrose, *C. marchica* as well as *S. schmidlei* adapted to heterotrophic conditions; this was evidenced by a decrease in duration of lag phase (not represented in the text) and by a better growth compared to non-adapted strains (Table II). Effect of sucrose on the light pre-treated, dark pre-treated (dark starved) and heterotrophic adapted strains of both the experimental organisms in relation to growth and nitrogen fixation was also studied. For dark starvation, both cyanobacteria were kept in basal inorganic medium in the dark for 30 days. The objective of the experiment was to investigate the response of an organism with different physiological status (with less endogenous substrates) to exogenously supplied substrates in the light and dark. Dark pre-treated strains, when transferred and grown in light with or without sucrose in the medium, increased the growth rate more than the light pre-treated and heterotrophic adapted strains. On the contrary, heterotrophic adapted strains of both cyanobacteria readily utilized the exogenous substrate (evidenced by increased dry weight yield) than the light pre-treated and dark pre-treated strains on dark incubation. However, a significant increase in the total nitrogen content of the cultures of *C. marchica* and *S. schmidlei* was obtained in the light as well as in the dark when inoculated with heterotrophic adapted strains (Table II). These results would suggest that the efficiency of exogenous carbon utilization by the cyanobacteria are the manifestations of the growth conditions provided to the organisms. It is possible that the physiological and biochemical status of the strains pre-treated or adapted in the dark would be different from that of the strains pre-treated with light. Light and dark treatments may also bring alteration(s) in membrane permeability and transport properties which could result in differential response of the test cyanobacteria to exogenous substrates during various growth conditions.

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EFFECTS OF HORMONES ON THE MULTIPLICATION OF THE HETEROTRICHOUS PROTOZON *BLEPHARISMA UNDULANS* STEIN

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The division rate of *Blepharisma undulans* is reduced by insulin treatment and increased by diiodotyrosine, diiodothyronin, histamine, serotonin or prednisolone treatment. In a later phase, the protozoa treated with histamine or prednisolone died. After insulin was deprived, the division rate strikingly increased as compared to the control cells, whereas the enhanced growth of the cells pretreated with any of the other hormones, except serotonin, continues in the absence of the hormone until the end of the observation period. The division rate of cells pretreated with serotonin decreased in the absence of the hormone. The cells of enhanced division rate hardly influenced by a second encounter with the same hormone, even insulin did not exert its characteristic negative effect. The fact that serotonin and insulin act in another way than do the other hormones suggests that the cell response is hormone-specific.

Hormonal imprinting ensues when a cell meets a hormone for the first time [1, 2]. Imprinting may occur when a hormone induces maturation of pre-programmed receptors and also when in lack of programmed receptor, the receptors (binding sites) are developed by hormonal effect from a non-specific membrane pattern. The former type is characteristic of organisms of higher order, while the latter of unicellular organisms. Protozoa have proved to be a good model for examining the mechanism of imprinting [3–5]. The majority of the tests have been performed by us, in populations of the holotrichous ciliate *Tetrahymena pyriformis*, strain GL.

The question has been raised whether the results obtained from experiments carried out with a single strain apply to unicellulars generally. To answer this question, we examined a number of *Tetrahymena* taxons. Each of the taxons responded to hormones, however, not uniformly [6–8]. Besides, perhaps independently of imprinting, hormones may modify (increase) the division rate of *Tetrahymena* taxons. This effect may last long, even through many generations [3, 9].

To check the general validity of the results obtained with the *Tetrahymena* model, we chose for further studies a unicellular of a different genus, *Blepharisma undulans* Stein.

B. undulans cells contain a red pigment, zoopurpurin. The cell's large size (150–300 μm) slower motion, and colour [10, 11] makes counting easy. Furthermore, cloning of selected *Blepharisma* cells meets no difficulties. Thus, *Blepharisma* is an ideal model for experiments of this type.

Materials and methods

Culturing. *B. undulans* cells were maintained in "Theodora" mineral water (Winecellars of Badacsony region, Kékkút, Hungary). The composition of the mineral water (mg/litre) is as follows. Cations: K^+ 23, Na^+ 45, NH_4^+ 0.28, Ca^{++} 277, Mg^{++} 57.6, Fe^{++} 6.1, Mn^{++} and Sr^{++} 3.8; anions: Cl^- 11, F^- 0.84, Br^- 0.1, SO_4^{--} 40, HCO_3^- 1244, BO_3^{--} 1.7. The "Theodora" water contains neither NO_3^- nor NO_2^- .

For culturing, one *Blepharisma* cell and a rice seed was placed in 10 ml of "Theodora" water. The unicellular consumed the bacteria cumulating on the rice seed. In these circumstances, i.e. without hormone, cells divided once daily. Culturing was performed at room temperature, the nutrient medium was changed every second day.

Hormone treatment. The following hormones were used: insulin (Insulin Semilente MC; Novo Copenhagen, Denmark, 10^{-6} M), DIT (3,5-diiodo-L-tyrosine; Fluka AG; Buchs, Switzerland, 10^{-8} M), T_2 (3,5-diiodo-D-thyronine; Ega Chemie, Steinheim, FR Germany, 10^{-8} M), prednisolone Na-succinate, (Di-adreson-F-aquosum, Organon, Oss, the Netherlands, 10^{-7} M), histamine dichloride (Reanal, Budapest, 10^{-6} M), serotonin creatinine sulphate (5-HT; Reanal, Budapest; 10^{-7} M).

The hormone-containing media were changed every second day.

Statistical analysis. The cells were counted daily. Significance was calculated by using Student's *t* test.

Results

Cells from both hormone-treated and untreated (control) cultures were transferred to hormone-free medium on the 7th day of experiment. After eight days (in other experiments 37 days) of culturing insulin-pretreated cells in hormone-free medium, cells were placed into medium containing insulin and cultured for eight days; other cells from the same cultures were examined without second hormone treatment. From cultures pretreated with any of the other hormones, cells were kept in hormone-free nutrient medium for 20 days and then treated for seven days with the same hormone again.

The cell division rate was markedly reduced by insulin (Fig. 1) and similarly increased by each of DIT, T_2 , histamine and 5-HT; only prednisolone appeared to be indifferent (Fig. 2). The cells cultured in medium containing histamine or prednisolone died by the 9th day. The multiplication of the cells kept in the presence of any of the other hormones grew constantly. Strikingly, the hormone effect did not appear immediately, its first appearance required 6 to 7 days. This means that *Blepharisma* responds slower than *Tetrahymena*. On the other hand, *Blepharisma* retains its new property once acquired as clearly shown by its behaviour after hormone treatment had been suspended or during repeated hormone treatment.

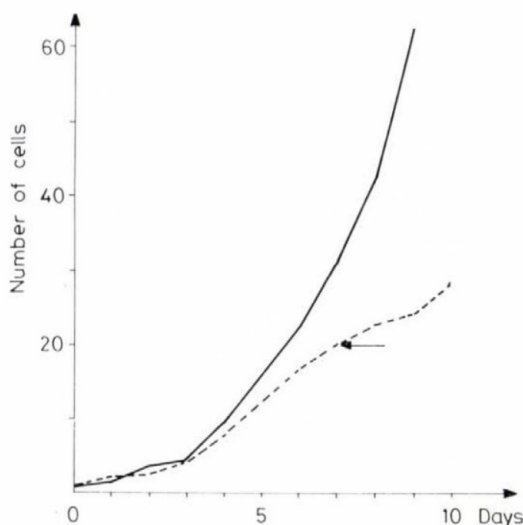


Fig. 1. Effect of insulin on the multiplication of *B. undulans* cell populations; ——— untreated control, - - - insulin-treated populations; arrow: $P < 0.01$ vs. control

If insulin-pretreated *Blepharisma* cells had been kept in hormone-free medium for 8 days, and were examined without any further hormone treatment, the pretreated cells multiplied significantly more intensely than the untreated controls (Fig. 3). A subsequent second insulin treatment induced no effect, neither positive nor negative (Fig. 3). The enhanced reproduction capacity of the insulin-pretreated cultures, on the other hand, was retained by the cultures grown in hormone-free medium for 37 days after hormone treatment (Fig. 4), indicating that the acquired property was fixed by the *Blepharisma* cells, which, however, partially lost their resistance to insulin, though, their multiplication rate did not fall below the control level on insulin effect.

The multiplication rate of cells exposed to any of the other hormones, except serotonin, and transferred to hormone-free medium on the 7th day was significantly higher than that of the control cells, even after 20 days of being kept in hormone-free medium. The multiplication of serotonin-pretreated cells lagged behind the control value. It was only T_2 that reduced the cell count considerably (Figs 5 and 6) at the second meeting as compared to the first meeting. The other hormones increased the cell count or left it unchanged.

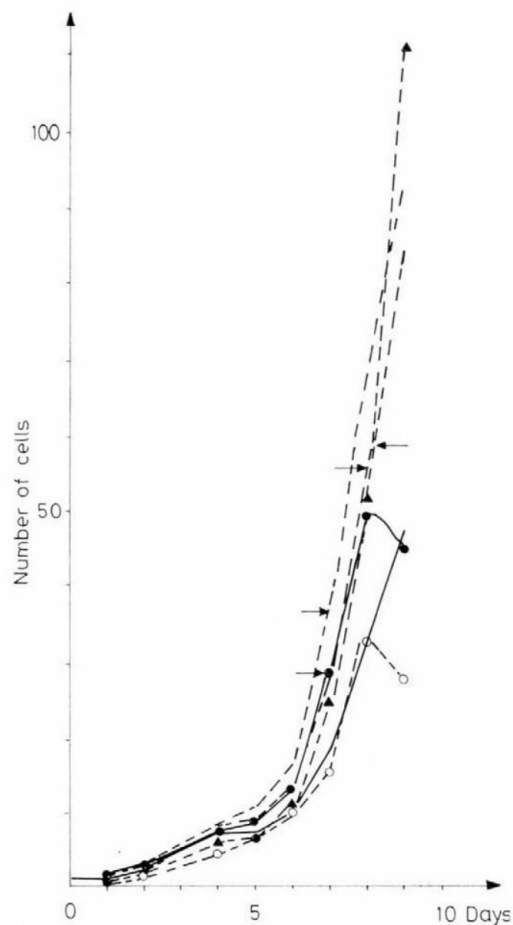


Fig. 2. Effect of four hormones on the multiplication of *B. undulans* cell populations; — untreated control, --- DIT-treated, -.-.- T_2 -treated, ●—● histamine-treated, ▲—▲ 5HT-treated, ○—○ prednisolone-treated cell populations; arrows: $P < 0.01$ vs. control

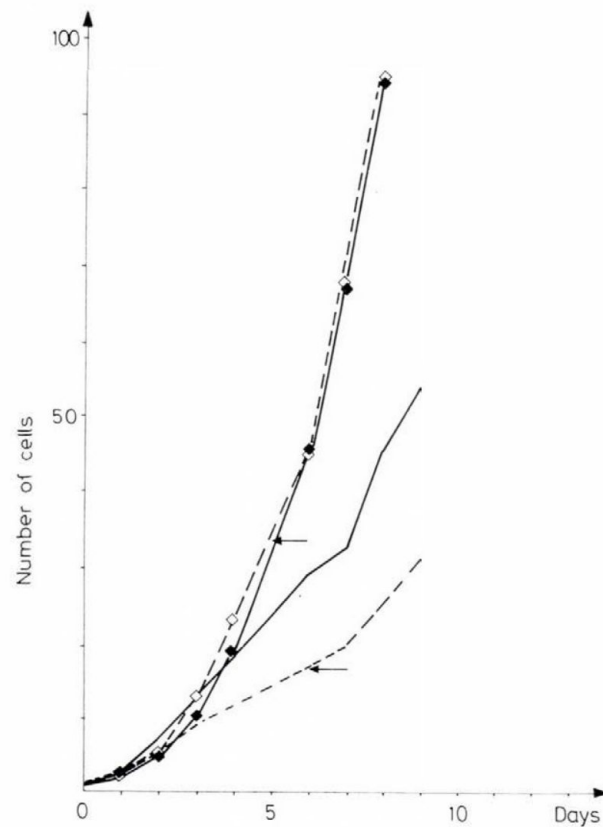


Fig. 3. Effect of a second incubation with insulin on the multiplication of *B. undulans* cell populations; — untreated control, $\triangle$ — $\triangle$ populations pretreated with insulin, then kept in hormone-free medium for 8 days and reincubated in insulin, — control cells without pretreatment, $\blacklozenge$ — control cells without second treatment; arrows: $P < 0.01$ vs. untreated control

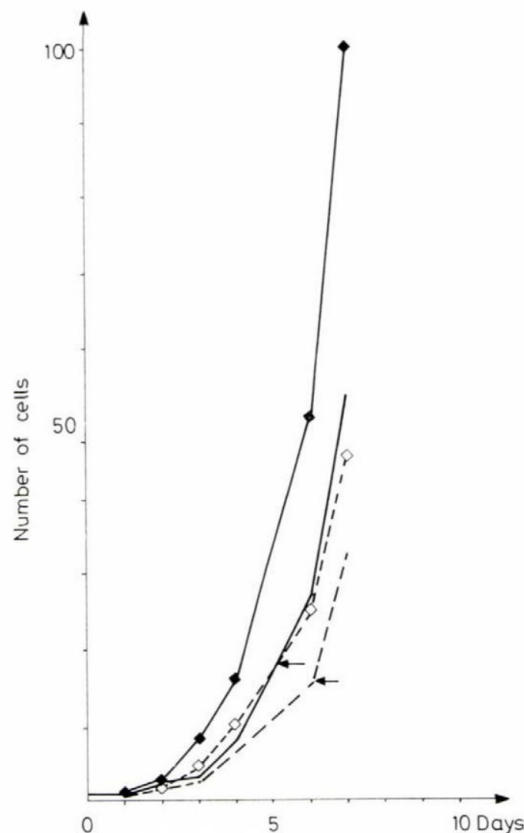


Fig. 4. Effect of a second incubation with insulin on the multiplication of *B. undulans* cell populations; — untreated control, $\diamond$ — $\diamond$ populations pretreated with insulin, then kept in hormone-free medium for 37 days and reincubated in insulin, --- control cells without pretreatment, $\blacklozenge$ — $\blacklozenge$ control cells without second treatment; arrows: $P < 0.01$ vs. untreated control

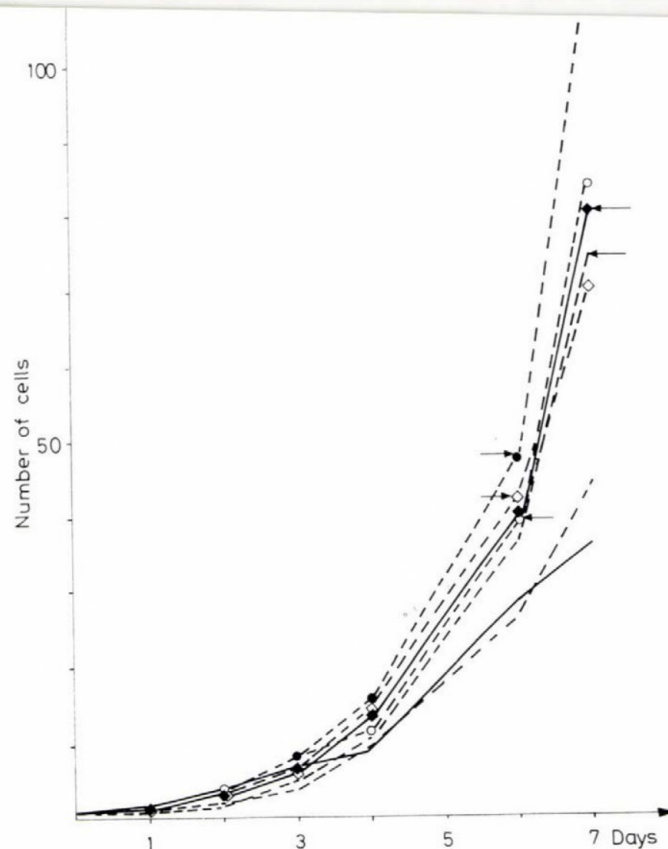


Fig. 5. Effect of a second incubation with hormone on the multiplication of *B. undulans* cell populations. The two periods of incubation in the presence of hormone were separated from each other by a 20-day period of incubation in hormone-free medium; — untreated control, $\blacklozenge$ — $\blacklozenge$ DIT pretreatment without reincubation with DIT, $\diamond$ — $\diamond$ DIT pretreatment and reincubation, --- T_2 pretreatment without reincubation with T_2 , -.- T_2 pretreatment and reincubation, $\circ$ — $\circ$ prednisolone pretreatment without reincubation with prednisolone, $\bullet$ — $\bullet$ prednisolone pretreatment and reincubation; arrows: $P < 0.01$ vs. untreated control

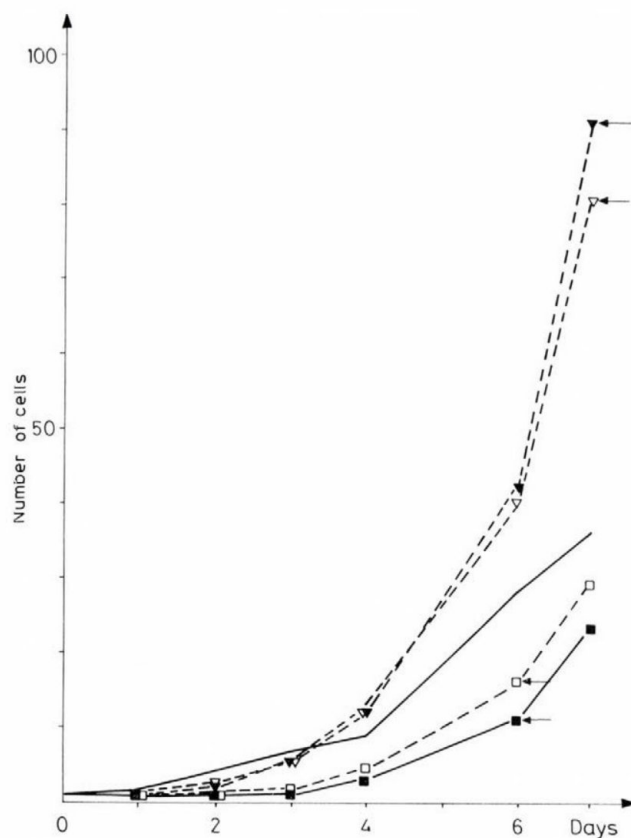


Fig. 6. Effect of a single incubation and a reincubation with hormone on the multiplication of *B. undulans* populations. The two incubations with hormone were separated from each other by a 20-day period of incubation in hormone-free medium; — untreated control, ■ — — ■ serotonin pretreatment without reincubation with serotonin, □ — — □ serotonin pretreatment and reincubation, ▼ — — ▼ histamine pretreatment without reincubation with histamine, ▽ — — ▽ histamine pretreatment and reincubation; arrows: $P < 0.01$ vs. untreated control

Discussion

It appears that, similarly to *Tetrahymena* [3, 9], *Blepharisma* is sensitive to hormone treatment; its cell division rate changes on hormone effect. The effect expressed in imprinting is, however, less pronounced than that expressed in a reduction of cell division rate. The latter is a lasting effect manifesting in progeny even after a number of generations [5]. We can not explain the cause of the difference in behaviour between *Tetrahymena* and *Blepharisma*. The *Blepharisma* cell, being visible with the naked eye, is much larger than the *Tetrahymena* cell; it is therefore possible that some membrane mechanisms of the two unicellulars are different. Furthermore, although in

Tetrahymena imprinting and the lasting "modification" of cell division ensue simultaneously and both follow the first meeting of cell and foreign material, it may be supposed that the two phenomena are independent of each other.

The response of *Blepharisma* to hormone treatment seems not be a sign of general responsiveness; this is shown by the diversity of the responses, viz., cell division rate was reduced by insulin, while it was increased by the other hormones and hormone-like substances under study. The diversity of the responses might be explainable by the variable hormone structures, viz., insulin, a polypeptide hormone, inhibited, while the amino-acid-type "hormones" and prednisolone (a steroid) enhanced the multiplication of *Blepharisma*. However, the "lasting modification" of the cell division rate following the discontinuation of hormone treatment shows that this is not an acceptable explanation, viz., among the hormone-treated cells those pretreated with the amino-acid-type "hormones" DIT, T₂ histamine behaved uniformly in the same manner as before, i.e. showed an enhanced multiplication, while those from a milieu containing serotonin multiplied slower than the control cells. Similarly, but with an inverse sign, the cells taken from the insulin-containing medium responded, in contrast with the immediate inhibiting effect of insulin, with a conspicuously enhanced multiplication, a response of long duration. It seems likely that the response induced by a hormone is characteristic of the inducer.

Our experimental results suggest that *B. undulans* is a better model than *Tetrahymena* for examining the "lasting modification" response, but it is a less suitable model for imprinting tests.

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DETERMINATION OF PHAGE TYPE, COLICIN TYPE AND PLASMID PROFILE IN *SHIGELLA SONNEI*

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Complex typing was carried out of 33 *Shigella sonnei* strains isolated in 1985 and 1986. One to six plasmids were carried by 32 strains typable by phages, whereas plasmid was not demonstrated in a phage-resistant strain. A 1.4 Md plasmid was carried by all strains. By determining the plasmid profile the clonal connection between the strains could be demonstrated. Strains assumed to be in clonal connection on the basis of phenotypic properties, were divided into subclones. Identical plasmid profile was observed among strains belonging to different phage and colicin types.

The methods used in molecular biology became simpler and this opened a possibility to their extended use in practice. The examination of plasmid DNA of bacteria important in epidemiology and in hospital hygiene gives a possibility to use a typing method independent of the phenotypic properties (serotype, bacteriocin production or immunity, biotype, antibiotic resistance pattern).

Determination of plasmid profile may clear up the source and spread of infection in nosocomial epidemics [1] caused by Gram-positive [2] and Gram-negative [3] bacteria. For facultatively pathogenic bacteria e.g. *Enterobacter cloacae*, frequently occurring in nosocomial infections — when other standardized typing methods were not available — examination of the plasmid DNA proved a suitable typing method [4]. Plasmid profile analysis was a proper typing method to subdivide some *Salmonella* serotypes [5, 6].

Bacterial dysentery is caused mainly by *Shigella sonnei* throughout the world and in Hungary, too. The phage and colicin typing carried out since 1966 was completed by determination of plasmid profile.

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Materials and methods

Bacterial strains. The 33 *S. sonnei* strains were isolated from human faeces according to standardized methods [7] in the laboratories of Public Health Stations. The geographical origin of the strains is listed in Table I-V. From the Hungarian National Collection of Medical Bacteria 10 *Shigella flexneri*, 10 *Shigella boydii* and 10 *Shigella dysenteriae* strains were obtained for examination.

Phage typing was carried out according to Hammarström [8] and modified by Kallings et al. [9].

Colicin typing. Abbott and Shannon's method was used.

Antibiotic sensitivity was determined to streptomycin (Sm), chloramphenicol (Cm), tetracycline (Tc), neomycin (Nm), kanamycin (Km), ampicillin (Ap), gentamicin (Gm), co-trimoxazole (CXT) and sulfamethazine (Su), by the use of "Resistest" disks (Human Institute for Serobacteriological Production and Research, Budapest), using modified Mueller-Hinton agar.

Plasmid isolation and analysis. Plasmid DNA was isolated according to Kado and Liu [11] and Birnboim and Doly's method [12] was used for restriction enzyme analysis. Restriction endonuclease EcoRI (New England Biolab) was used as recommended by the supplier.

Agarose gel electrophoresis and estimation of plasmid size was performed as described by Meyers et al. [13]. Vertical electrophoresis was carried out in 0.7% Sigma agarose gel at 35 mA, 125 V for about 3 h and then the gel was stained with ethidium bromide (1 mg/1000 ml) for 30 min; after the removal of the ethidium bromide, the preparations were photographed under short-wave UV light. Electrophoresis of the digested samples was carried out in horizontal slab gel using 0.8% agarose for about 16 h.

Molecular weight standards. *Escherichia coli* V517 [14]: 1.4, 1.8, 2.0, 2.6, 3.4, 4.8, 35.8 Md; R27: 112 Md; R1: 62 Md; pSP1: 140 Md (from Dr. T. Pál, Institute of Microbiology, University Medical School, Pécs).

Plasmid elimination experiments were carried out with ethidium bromide [15], acridine orange [16] at 42 °C for 72 h. In each experiment 10 isolated colonies were subjected to gel electrophoresis.

Conjugation was performed as described by Tschäpe et al. [17].

Results

Tables I-V show the phage and colicin types of the strains, their sensitivity to antibiotics, the size of the carried plasmids and the origin of the strains. The cultures, except strain 333 in Table V were isolated in the year 1985.

The results are grouped to demonstrate the connection between the phenotypic properties and plasmid profiles. Table I shows plasmid profiles of strains belonging to the same phage- and colicin type. Table II presents data of strains isolated from the same locality at different occasions, possessing the same plasmid profile and showing minimal differences in their lytic patterns. Out of the applied ten *S. sonnei* type phages the same lytic degree was given by nine phages, therefore only the lytic reactions obtained by phage I are presented.

Antibiotic sensitive, non-colicinogenic strains belonging to different phage types are shown in Table III(a). A 1.4 Md plasmid was carried by these strains.

Table III(b) shows data for strains of phage/colicin type 87/0 and 2/4, respectively. Out of the five strains four were resistant to Sm Ap CXT and one was resistant to Sm Ap. The five strains, derived from three counties

Table I*Plasmid profiles of antibiotic sensitive S. sonnei strains belonging to the same phage and colicin type*

Designation	Phage type	Colicin type	Antibiotic sensitivity	Plasmid molecular weight (Md)					Origin of the strains	
									county	locality
(a) 262	32	4	sensitive	65	1.4				Tolna	Bonyhád
263	32	4	sensitive	65	1.4				Tolna	Németkér
315	32	4	sensitive	65	1.4				Zala	Vindornyafo
(b) 312	6	0	sensitive	75	23	4.2	1.6	1.4	Tolna	Sárszentlőrinc
314	6	0	sensitive	75	23	4.2	1.6	1.4	Tolna	Szekszárd
447	6	0	sensitive	75	23	4.2	1.6	1.4	Tolna	Pincehely
483	6	0	sensitive	75	23	4.2	1.6	1.4	Békés	Gyula
(c) 316	7	0	sensitive	1.4					Zala	Lenti
317	7	0	sensitive	1.4					Zala	Lenti
318	7	0	sensitive	1.4					Zala	Lenti
319	7	0	sensitive	1.4					Zala	Lenti

Table II*Complex typing of a family outbreak caused by S. sonnei*

Designation	Phage type	Lysis by phage I	Colicin type	Antibiotic sensitivity	Plasmid molecular weight (Md)				Origin of strains	
									county	locality
140	Nb*	++++	0	sensitive	96	80	5.0	1.4	Győr-Sopron	Győr
142	13	—	0	sensitive	96	80	5.0	1.4	Győr-Sopron	Győr
160	Nb*	++++	0	sensitive	96	80	5.0	1.4	Győr-Sopron	Győr
161	Nb*	++++	0	sensitive	96	80	5.0	1.4	Győr-Sopron	Győr

* Not characteristic

Table III*Phage type, colicin type, antibiotic resistance and plasmid profile of S. sonnei strains*

Designation	Phage type	Colicin type	Antibiotic resistance	Plasmid molecular weight (Md)				Origin of strains	
								county	locality
(a) 440	65	0	sensitive	1.4				Heves	Gyöngyös
212	13	0	sensitive	1.4				Szolnok	Kenderes
316	7	0	sensitive	1.4				Zala	Lenti
(b) 256	87	0	Sm Ap CXT	72	4.0	1.4		Szabolcs-Szatmár	Tiszavasvári
257	87	0	Sm Ap CXT	72	4.0	1.4		Szabolcs-Szatmár	Tiszavasvári
373	87	0	Sm Ap	72	4.0	1.4		Győr-Sopron	Hegyeshalom
473	2	4	Sm Ap CXT	72	4.0	1.4		Tolna	Dombóvár
624	2	4	Sm Ap CXT	72	4.0	1.4		Tolna	Dombóvár

differed both in phage and colicin type but the carried plasmids were the same in number and size. After digestion with EcoRI the fragments differed in size and in number in the case of strains belonging to different phage and colicin types.

The plasmid profiles of strains isolated from minor local outbreaks are presented in Table IV(a) and (b). The phenotypic properties were the same but two strains carried additional plasmids.

Table V shows the plasmid profiles of phage type 87 non-colicinogenic strains resistant to Sm Ap Su and Sm Ap CXT, and the plasmid profile of an antibiotic sensitive strain of phage/colicin type 87/12. The 72 Md R plasmid

Table IV

Differentiation of antibiotic sensitive S. sonnei strains belonging to the same phage and colicin type according to their plasmid profile

Designation	Phage type	Colicin type	Antibiotic sensitivity	Plasmid molecular weight (Md)				Origin of strains			
								county	locality		
(a)	164	7	0	sensitive	23	3.2	1.4	Zala	Zalabaksa		
	165	7	0	sensitive				Zala	Zalabaksa		
	220	7	0	sensitive				Zala	Zalabaksa		
	221	7	0	sensitive				Zala	Zalabaksa		
(b)	714	3	0	sensitive	70	54	4.0	3.0	Baranya	Keresztespuszta	
	715	3	0	sensitive	70	54	42	22	4.0	3.0	Baranya

Table V

Plasmid profiles of non-colicinogenic, Sm Ap Su and Sm Ap CXT resistant S. sonnei strains belonging to phage type 87

Designation	Phage type	Colicin type	Antibiotic resistance	Plasmid molecular weight (Md)				Origin of strains	
								county	locality
373	87	0	Sm Ap Su	72	4.0		1.4	Győr-Sopron	Hegyes-halom
553	87	0	Sm Ap Su	72	4.0		1.4	Tolna	Paks
595	87	0	Sm Ap Su	72	5.6	4.0	1.4	Pest	Budapest
719	87	0	Sm Ap Su	72		22	4.0 1.6	1.4 Tolna	Pusztahencse
333 (1986)	87	0	Sm Ap Su	72	4.0		1.4	Pest	Budapest
256	87	0	Sm Ap CXT	72	4.0		1.4	Szabolcs-Sz.	Tisza-vasvári
257	87	0	Sm Ap CXT	72	4.0		1.4	Szabolcs-Sz.	Tisza-vasvári
365	87	12	sensitive	65	44	18	1.4 Pest		Tápió-szele

determined resistance uniformly to Sm Ap Su. There was a difference between the plasmid profiles of strain 87/12 and strains 87/0. Plasmid DNA was not demonstrable in one untypable strain isolated in 1986 (not shown in Table V).

Plasmid elimination experiments were carried out with strains 220, 221, 316 and 317, belonging to phage/colicin type 7/0; the probably cryptic, 1.4 Md plasmid could not be eliminated by any of the three methods.

The plasmid profiles were examined of *S. flexneri* strains isolated from patients and of 10 *S. dysenteriae*, 10 *S. flexneri* and 10 *S. boydii* reference strains received from the Hungarian National Collection of Medical Bacteria. The 1.4 Md plasmid was not demonstrated in any of the four *S. flexneri* patient strains, whereas it was present in serotype 1 and 7 *S. dysenteriae*, in serotype 4b and 5 *S. flexneri*, in serotype 6 and 8 *S. boydii* and in two *S. sonnei* phase I strains. A 120 Md plasmid characteristic of *S. sonnei* phase I [18] was also shown in the two latter cultures.

Discussion

Phage types of *S. sonnei* strains sent in the year 1985 to the Phage Department of the National Institute of Hygiene were, in the order of frequency, the following: 3 (20.9%), 2 (19.9%), 65 (12.6%), 6 (9.4%), 7 (7.1%), 32 (3.7%), 87 (2.9%), 13 (2.7%), not-characteristic (1.4%). For our experiments, with a few exceptions non-colicinogenic, antibiotic sensitive strains belonging to different frequent or rare phage types were chosen.

The determination of plasmid profile proved to be a valuable tool for epidemiological purposes. The clonal connection was demonstrated in three cases by determining the number and size of the plasmids carried by strains that seemed to be clones on the basis of their phenotypic markers (Table I).

Plasmid profile analysis verified the common source of, and clonal connection between, phage type 13 and not characteristic strains isolated from a family outbreak (Table II).

Two examples were found for those rather rare cases, when the plasmid profiles were the same, though the phenotypic characters differed. As shown in Table III(a), antibiotic sensitive, non-colicinogenic strains belonging to phage types 65, 13 and 7 harboured uniformly a 1.4 Md plasmid. Table III(b) demonstrates that plasmids of the same number and size were carried by antibiotic resistant strains of different phage/colicin types, isolated from three counties. The additional plasmids present in strains 165 and 715 were probably acquired from the environment, but another possible explanation may be that they had been eliminated from the other strains as a consequence of certain environmental effect.

There were but slight differences in the number and size of the plasmids carried by the Sm Ap Su resistant strains of phage/colicin type 87/0 (Table V).

We could not demonstrate the large (120 Md) virulence plasmid, characteristic of phase I of *S. sonnei* [18] in the cultures examined, because these strains were of phase II; however, the 120 Md plasmid was found in the two phase I reference strains obtained from the Culture Collection.

Tacket et al. [19] examining plasmid profiles of *Shigella* species derived from Bangladesh, found the method valuable for epidemiological purposes. They proved the spread of different clones in areas hyperendemic for dysentery.

Tietze et al. [20] studied the spread and clonal character of multiple resistant *S. sonnei* strains in GDR. Plasmids, similar in number and size to our findings, were carried by their *S. sonnei* strains.

Holmberg et al. [21] studying *Salmonella typhi-murium* epidemics found plasmid profile analysis as significant as phage typing. Plasmid profile analysis can complete phage typing and, in lack of adequate phage and colicin typing methods, may be considered as a valuable substitute to them.

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TENTH CONGRESS OF THE
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ABSTRACTS OF PAPERS AND POSTERS

Symposium I

Biotechnology and Industrial Microbiology

THE JANUS-FACE OF BIOTECHNOLOGY: SCIENCE AND PROFIT-MAKING

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Whereas a concise definition of biotechnology is rather difficult, it is widely accepted that "Biotechnology is an integrated application of the results of biological, chemical and engineering sciences to obtain any kind of marketable endproducts from suitable raw materials". Biotechnology may be regarded more than pure science, concerning also markets and commercial application. It is a somewhat peculiar blend of science and finance. Two strategic routes are outlined for biotechnologist: one for finding the technology that can be commercialized and one for finding the financial resources to turn a discovery into a business. The industrial and commercial importance of biotechnology is rapidly increasing, especially in large scale fermentation, in traditional industries in the fields of pharmaceutical-chemistry, foodstuffs and environmental protection. The market for biotechnology is expected to grow at the rate of 6–8% per annum. It needs close cooperation between research establishments and industry in view of a direct transfer of research and development results to industrial applications.

ELECTROPORATION DEPENDENT TRANSFORMATION OF LACTIC ACID BACTERIA

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Lactic acid bacteria are industrially important species and are widely used in dairy fermentations. A genetic transformation system similar to the one functional in competent cells of clinically important streptococci is absent in lactic acid bacteria, which, in part, has contributed to the lack of progress

in the development of recombinant DNA techniques for these microorganisms. Exposure to a pulsed electric field results in the transient permeabilization of cells allowing the uptake of DNA through pores created in the plasma membrane. Electroporation has been used to introduce DNA into plant protoplasts, and animal cells. The technique also facilitates DNA uptake by protoplasts of *Saccharomyces cerevisiae* and *Bacillus cereus* as well as by intact cells of *S. cerevisiae* and *Escherichia coli*. In this paper we describe the electroporation-dependent uptake of plasmids up to 30KB in molecular mass by different species of lactic bacteria and the effect of experimental conditions of the transformation process. Transformation of streptococci and lactobacilli by electroporation is a useful technique in the genetic development of these microorganisms.

PENICILLIUM CHRYSOGENUM, ITS GOLGI APPARATUS AND PENICILLIN PRODUCTION

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In a high-yield mutant of *Penicillium chrysogenum* PQ-96 cells the benzylpenicillin is synthesized, accumulated and transported beyond the cytoplasm in Golgi organelles. The enzymes taking part in biosynthesis of benzylpenicillin: delta (L-alfa-aminodipyl)-L-cysteinyl-D-valine, ACV synthetase, the isopenicillin N synthetase (cyclase), side chain CoA ligase and acyl-transferase were found to be present in Golgi vesicles. By means of immobilized Golgi organelles in a well defined system we succeeded in total cell-free biosynthesis of benzylpenicillin in vitro.

BREEDING OF BREWER'S YEAST: IMPROVEMENT OF FLOCCULATION AND TRANSFER OF KILLER FACTOR

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The main aim of strain improvement program is to construct yeast strains which are more suitable for a given beer fermentation technology whereby quality of beer is improved and economy of production is increased. An attempt was made to decrease the production of undesirable aromas (vicinal diketones), to increase the flocculation ability as well as to construct

yeast strains with killer activity for the purpose to repress the contaminating wild yeasts. During the experimental work mutagenic treatment and the method of protoplast fusion were used and by means of the latter new strains of increased flocculation and of killer factor production were constructed from which brewer's yeast strains with good fermentation ability were selected.

DELETION OF VECTOR DNA SEQUENCES IN CLONING OF A 2 μ DERIVATE PLASMID OF *SACCHAROMYCES CEREVISIAE* RXII

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Systematic study of the mitochondrial DNA (mtDNA) of the *Saccharomyces cerevisiae* strain RXII revealed that the 10 000 g membraneous-mitochondrial pellet, the source of mtDNA, contained two other extrachromosomal genetic elements. One of them was resolved as a 6.2 kilobasepairs (kbp) *Eoi*RI fragment in agarose gel electrophoresis and hybridized to the part of a 2 μ probe. The 6.2 kbp band was isolated from the gel, ligated into the *Eco*RI site of the pBR328 vector and the *Escherichia coli* HB101 strain was transformed. The restriction mapping of the chloramphenicol sensitive plasmids showed that the vector sequences, between the ampicillin and tetracyclin resistance gene promoters were deleted. This event was not detected when *Eco*RI linearized vector DNA was ligated and transformed: all of the clones recovered were chloramphenicol resistant. These data suggested that cloning of the entire 2 μ derivate DNA of strain RXII gave room for expression of gene(s) encoded by the inserted DNA, which resulted in a such a harsh recombination event in a *recA13* background.

HYBRIDIZATION AND BREEDING OF GLYCOAMYLASE PRODUCER *ASPERGILLUS* *NIGER* STRAINS VIA PROTOPLAST FUSION

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Glycoamylase producer *Aspergillus niger* strains derived from various sources possess interesting common characteristics which have alternative proline or arginine requirements. Following mutagenic treatment (UV, NTG)

single proline or arginine requiring strains could be isolated, but these auxotrophic markers were not complementing in attempted crosses. Strains carrying additional nutritional requirements, like cysteine, leucine, lysine, adenine, cytosine, PABA were also isolated. These markers proved to be complementing characters in crosses. Auxotrophic mutants derived from various parental sources in all possible combinations were hybridized via protoplast fusion. Protoplasts can be released from surface growing cultures by using induced trichoderma-enzyme. Fusion experiments were performed by 25% PEG 4000 with 0.1 M CaCl_2 . From all attempted crosses fusion products were recovered. At the beginning of regeneration they proved to be heterokaryons. Prototrophic hybrids — possible diploids — were isolated from the heterokaryons. Using benzimidazole derivatives as haploidizing agents prototrophic hybrids produced sectors showing parental and recombinant segregation. The practical aim of this work is to select for prototrophic haploid recombinants and screen them for glycoamylase production in submerged condition.

ELECTROFUSION OF *ASPERGILLUS NIDULANS* PROTOPLASTS

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Electric field-induced fusion was carried out between two auxotrophic mutants (ade^- and lys^-) of *Aspergillus nidulans*. Close membrane contact between the protoplasts was achieved by dielectrophoresis in an inhomogeneous alternating field (0.8 kV/cm field strength and 10 s duration). Due to dielectrophoresis, pearl chains of two strains are formed between the electrodes. Protoplast fusion was induced by application of a single square field pulse sufficiently high to induce reversible breakdown in the membrane contact zone between cells within a pearl chain (3.3 kV/cm, 40 μs). Genetic complementation of the auxotrophic strains indicated the occurrence of fusion. In the case of electrofusion 7 to 20 times more heterokaryotic colonies were grown as compared to fusion.

CONSTRUCTION OF GENETICALLY MARKED RUMEN MICROORGANISMS AND OPTIMALIZATION OF THE RUMEN FERMENTATION

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With respect to meat and milk production, the supply and proportion of acetic acid and propionic acid biosynthesized by the rumen microorganisms play an essential role. Samples were taken from the rumen of a sheep fed on a given feedstuff. Microorganisms producing in vitro acetic and propionic acid were isolated. The selected strains were marked genetically by plasmid DNA transformation and their ruminal growth and propagation were examined on selective media. A strain that was able to grow in the rumen at least for 60 days and could ferment carbohydrates to propionic acid was isolated and orally administered to ruminants for developing and modulating the rumen culture. A preparation made from the isolated microorganism markedly stimulated weight gain in sheep fed on a given feedstuff.

HOST PLANT DEPENDENT FUNCTION OF NODULATION REGULATORY GENES IN RHIZOBIUM

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The *Rhizobium* genes inducing root nodules for symbiotic nitrogen fixation (*nod* operons) are switched on by the regulatory *nodD* gene, which acts in conjunction with plant-derived signal molecules (flavonoids). In the case of *Rhizobium meliloti*, three *nodD* loci are present in the genome. We have investigated the involvement of these *nodD* copies in the nodulation of different host plants. Two approaches were employed in our studies: (i) the *nod* operon inducing ability of the *nodD* copies in conjunction with several host plant extracts (using *nodC-lacZ* fusions); (ii) effects of mutations in particular *nodD* copies on the nodulation. We have shown that the *nodD* genes are allelic forms which recognize different types of flavonoids. Only *nodD* genes for which the actual host plant provides the flavonoids suitable to interact with can contribute to the activation of the *nod* operons. Thus, the *nodD*

content of the rhizobia as well as the flavonoid composition of the particular hosts influences the efficiency of nodulation. Modifications of these two factors may provide the possibility of improving the competitiveness of *Rhizobium* in establishing symbiotic association with the host plant.

CLONING AND EXPRESSION OF TUMOUR NECROSIS FACTOR GENE IN *ESCHERICHIA* *COLI*

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We isolated a phage containing the human tumour necrosis factor gene by using an oligonucleotide probe and constructed the physical map of the phage genom. Then the coding sequence of the tumour necrosis factor gene with a synthetic DNA fragment was cloned into an expression vector. The recombinant bacterium produced biologically active tumour necrosis factor.

DISTRIBUTION OF METHANOGENIC ASSOCIATIONS IN VARIOUS ECONICHES

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One hundred and sixty samples of natural substrates taken from various econiches were studied for the presence of anaerobic microorganisms able to convert wastes of cattle-breeding and poultry-raising in biogas. Methane formation was observed not only in traditional methane-generating bacteria habitats as silt of fresh water reservoirs, but in sediment of salt water reservoirs (e.g. the Black and White seas) as well. Differences of microbial methanogenic activity in various econiches were determined.

ELIMINATION OF ENVIRONMENT-POLLUTING HYDROCARBONS USING BIOTECHNOLOGY

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The objective of this study was to develop a biotechnological process for elimination of hydrocarbon contaminations. Hydrocarbon decomposing microbe-cultures with the predominance of *Nocardia*, *Pseudomonas*, *Flavobacterium* and *Candida* spp. were isolated from soil samples contaminated with oil. The inoculation with a starter-culture prepared from hydrocarbon decomposing strains has been found to be effective in the laboratory and small plot experiments and under large-scale application conditions. Since in Hungary the most important problems are oil-polluted waters and sludges due to activities of various factories, we have developed a method for eliminating the permanently formed oil contaminations. At first the oil is separated from the oily waste water then, the water acceptable can be used for irrigation or it can be transferred into canals. The oily part together with sludges supplemented with additives is transferred into well-isolated places in 20–40 cm layer, and inoculated. The starter-culture was made by non-sterile fermentation on the spot. Recently there is a model plant under development for optimization of the technological processes and for establishment of an integrated system.

IN VIVO RECOMBINATION IN FAST GROWING MYCOBACTERIUM STRAINS

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An effective method had been developed for spheroplast preparation from fast growing mycobacteria. Mycobacteria were grown in a shake flask culture medium at 32 °C. Spheroplasts were prepared by lysozyme treatment of vegetative cells after cultivating in a glycine and vancomycin containing medium. Mutants of mycobacteria (Ade⁻; Val⁻; Pro⁻ and Str^R) were obtained by the chemical mutagenesis of the wild type strains with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidin. Spheroplasts of the mutants mixed in 1:1 ratio were treated 50% (w/v) polyethylene glycol 6000 for 5 min at 37 °C. The recom-

binants were selected on the basis of genetic markers. The recombination frequency was 5.6×10^{-2} to 1.6×10^{-3} . In vivo genetic recombination is a useful method to improve industrially important *Mycobacterium* strains.

FORMATION OF P-HYDROXY-PHENOXYACETIC ACID AND P-HYDROXY-PHENOXYMETHYL-PENICILLIN IN PENICILLIN-V FERMENTATION

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Hydroxylation of precursor and side-chain hydroxylated penicillin formation cause a considerable loss of the precursor and of the end-product penicillin, respectively. In phenoxymethyl-penicillin fermentation the precursor is partially hydroxylated to p-hydroxy-phenoxyacetic acid and p-hydroxy-phenoxy-methyl-penicillin formed. The formation of hydroxylated by-products was studied in shaking culture experiments using an industrial high-producer *Penicillium chrysogenum*. Exogenously added penicillin-V was not hydroxylated in fermentation run on precursor-free broth. The same penicillin production was found using p-hydroxy-phenoxyacetic acid or phenoxyacetic acid as a sole precursor. In precursor-competition experiments where both the phenoxyacetic acid and its p-hydroxy derivative were available as precursors for penicillin biosynthesis, phenoxyacetic acid was found to be the preferred precursor, and the rate of p-hydroxy-phenoxy-methyl-penicillin formation was hardly affected by the level of p-hydroxy-phenoxyacetic acid in the presence of phenoxyacetic acid. Changing the pH of the broth either by the nutrient composition or by pH adjustment the p-hydroxylation of phenoxyacetic acid was drastically increased by lower pH. Surprisingly, the percentage of formed p-hydroxy-phenoxy-methyl-penicillin in the total penicillin formed was not enhanced by the lower pH. It is suggested that the formation of the hydroxyl group appearing in the p-hydroxy-phenoxy-methyl-penicillin takes place during the biosynthesis and/or excretion of penicillin-V.

ANTIBACTERIAL ACTIVITY OF THE MICROFLORA OF INSECT PATHOGENIC NEMATODES

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Bacterial symbionts isolated from insect pathogenic nematodes showed antimicrobial activity, not only in agar medium but also in submerged culture. The antibacterial activity of the filtrate was tested by agar diffusion method with *Bacillus subtilis*. All isolates were resistant against the antibacterial activity. Antibacterial agents may be produced in living nematodes by symbiotic bacteria.

"CORROSION" OF METAL-INSTRUMENTS BY MICROBES

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In the emulsion-system of an iron industrial company, in the course of recirculation, microorganisms cause "black spots" ("corrosion") on iron-tools. In the contaminated system the bacteria occur in high counts and exist for a long time. The process is enhanced by the temperature optimum for their proliferation and survival. The agents responsible are *Clostridium* species showing an increased resistance to antiseptics.

A HIGHLY SENSITIVE PENICILLIN ELECTRODE

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Bacillus cereus was grown in a complex medium (tryptone broth) shaken at 37 °C. The formation of penicillinase was induced by benzylpenicillin, and measured by iodometry. The extracellular enzyme was adsorbed on Celite 545 at pH 7.0. The enzyme was eluted with Tris-citrate buffer by the pH gradient method. The partially purified enzyme was immobilized by entrapping in poly-acryl-amide gel on the surface of a pH-sensitive glass electrode.

A linear relationship was observed between the electrode potential and the concentration of penicillin between 0.2–10 mM/litre. The electrode potential was reproducible within $\pm 5\%$. The sensitivity of penicillin electrode was stable, showed no decrease in 2 months.

THE APPLICATION OF BACTERIAL PROTEINASES AS ENZYMATIC COMPONENTS OF WASHING POWDERS

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We have compared the enzymic preparation PBS (component of the washing powder "E-automat") obtained from *Bacillus* sp. B-3 and the commercial proteinase "Maxatase" (Gist-Brocades-Firm). Proteolytic activity of the both enzymes preparation (P) was compared by means of Anson and Delft methods. The preparations had nearly constant enzymatic activity at 60 °C at pH 6–12.0 (B-3 1450 U, Maxatase 1000 U). Both had a similar sensitivity to washing powder components (borax, polyphosphates and Na_2SO_4) which decreased their enzymatic activity. The ability of washing bleaching of "E-automat" in relation to test texture "EMPA-116" (contaminated with blood, milk and ink) was similar. The washing powder "E-automat", containing our preparation PBS, showed a greater detergency for textures "EMPA-101" and "EMPA-112" than "Maxatase".

INFLUENCE OF ADAPTATION ON THE FERMENTATION SPEED OF IMMOBILIZED *SACCHAROMYCES CEREVISIAE* V30

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In bioreactors continuously producing alcohol, the number of living cells in the fixed phase gradually decreases. Actually, the cell number determines the half life of the immobilized biomass. The change of fermentation speed by yeast cells adapted or non-adapted to high concentration of sucrose (25%) and ethanol (5%) was investigated. Yeast cells were closed in alginate

gel bed and fermented in beet sugar molasses containing 15% sucrose. From the beginning of second day there was a significant difference between the adapted and non-adapted cells. The adapted cells fermented 3–6-fold faster as compared to the non-adapted ones. Furthermore, at the end of the fermentation, in the adapted cell culture the living cell number was 30 times higher than in the control cell culture.

Symposium II

Biology of Interferon System

STUDIES ON THE INTERFERON SYSTEM IN SLE

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An occasional appearance of interferon (IFN) in the sera of patients with SLE often alternates with the appearance of a neutralizing activity named tentatively "anti-IFN". While the IFN found in SLE patients seems not to differ from an acido- and termolabile fraction regularly found in leukocyte-derived IFN preparations, the anti-IFN has a mol wt of about 30 000, reacts only with alpha type IFNs and, with swine anti-Ig serum. This suggests that it might represent a light IG-chain. The IFN activity found in sera shows a correlation with the activity of the disease. While anti-IFN is also found in highest percentage (57%) in the sera of patients with active disease, it may be present also in pharmacologically suppressed or inactive disease stages. An IL-2 activity was also found in about 15% of SLE patients.

ANTIVIRAL ACTIVITIES OF INTERFERONS

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In addition to modulating cell growth and the immune system, the interferons are also antiviral agents. Their antiviral activities appear to be mediated by proteins whose synthesis they induce. This induction is a consequence of an interaction of the interferons with specific cell surface receptors and the resulting enhanced expression of a set of interferon-responsive genes. Several such genes have been cloned. Some of these have been shown to include DNA segments located at the 5' terminal regions which make the expression of the genes responsive to interferon. Among the proteins specified by these genes are several with antiviral activities. These include e.g. a family of 2'-5' oligoadenylate synthetases located in various subcellular compartments, RNase L and a double stranded RNA activatable protein kinase. There is

strong evidence supporting the involvement of these enzymes in blocking the replication of particular viruses. Some viruses have specific gene products to overcome the effects of some of these enzymes (e.g. adenoviruses specify VA RNA which blocks the activation of the protein kinase). Another interferon induced protein is designated as Mx. This impairs specifically the replication of influenza virus. Much remains to be learned about the various antiviral mechanisms of the interferon system.

INOSITOL PHOSPHOLIPID TURNOVER AND PROTEIN KINASE C TRANSLOCATION ARE STIMULATED BY POLY I : C IN HUMAN AMNION CELLS (UAC)

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Poly I : C induces mRNA synthesis of beta interferon (IFN) and 2'-5' oligoadenylate synthetase similarly to platelet derived growth factor (PDGF). In addition, both PDGF and IFN activate the inositol phospholipid breakdown induced signal transduction mechanism. On the bases of these data it was postulated that (i) poly I : C per se has IFN-like effects (for example antiviral effect), (ii) activates inositol phospholipid breakdown and (iii) induces protein kinase C activity. The experiments performed proved that (i) using a VSV-UAC system poly I : C has an antiviral effect even in the absence of IFN synthesis, (ii) measuring ^3H -myoinositol incorporation into inositol phospholipids, poly I : C transiently activates inositol phospholipid turnover and (iii) poly I : C induces protein kinase C translocation from the cytosol to the membrane bound fraction. All these findings comprise strong evidence that the major pathway of induction and mode of action of IFN are realized through common signal transduction mechanisms of the cell.

SYNERGISTIC ANTIVIRAL EFFECT OF INTERFERON AND VITAMIN A

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IFN-s and retinoids are known to regulate several biological functions in different cell types. The combination of these substances results in the potentiation of their antiproliferative effect. In addition, 2'-5'-oligoadenylate synthetase, an enzyme playing an important role in the antiviral activity of IFNS-s, can be also induced by retinoic acid. Measuring viral (VSV) RNA synthesis by the use of human amnion cell cultures, it was found that vitamin A enhanced the inhibitory effect of IFN-alpha. It was also shown that vitamin A transiently activates ³H-myoinositol incorporation into the water soluble inositol 1-phosphate and induce the translocation of protein kinase C. These findings and our earlier results suggest that the antiviral state of the cells can be activated by different substances (i.e. IFN, poly I : C, PMA, vitamin A), which trigger the breakdown of inositol phospholipids and activate the association of protein kinase C to the membrane.

IFN: SARCOLECTINS IN COORDINATED GROWTH. REGULATION OF THE EXPRESSION OF PROTO-ONCOGENES AND ONCOGENES

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During the fetal phase of pregnancy, alpha or beta interferons are continuously excreted in the placenta. Antagonists (TAI), presently named sarcolectins, are responsible for the decay of the interferon functions previously induced. We postulate that (as shown by others) growth factors induce interferons as feedback loops for a single growth cycle. The addition of sarcolectins allows us to restore their initial status to the cells, making them available to respond to new stimuli. This stepwise development of growth probably plays a role in regulating the expression of proto-oncogenes, as well as occasionally oncogenes. This could contribute to the understanding of why prolonged interferon treatment can be in some cases promote stable reversion of cancer cells to non-malignancy.

DEPRESSION OF POLY I : C-INDUCED INTERFERON PRODUCTION DURING PROGRESSION OF TRANSPLANTABLE MURINE TUMOURS

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Poly I : C induced serum interferon (IFN) levels were determined in healthy and tumour-bearing mice 2 h after intraperitoneal injection of the inductor (100 μ g per mouse). Healthy female Balb/c and C57B1/6 mice produced consistently higher serum IFN levels than female DBA/2 mice. Syngeneic mice were transplanted with the following tumours: SP4 spontaneous adenocarcinoma (Balb/c), BaFl benzyrene-induced fibrosarcoma (Balb/c), Lewis lung carcinoma (C57B1/6), P815 mastocytoma (DBA/2) and P388 leukaemia (DBA/2). The levels of poly I : C induced serum IFNs were measured at different intervals after tumour transplantation. The level of the induced IFN decreased as the mean diameter of intramuscularly transplanted SP4, BaFl and Lewis lung tumour increased. In DBA/2 mice bearing P815 or P388 tumours growing in ascitic form, the level of induced IFN was also reduced. This phenomenon was also observed when P815 and P388 tumours were inoculated intramuscularly. Thus, it can be stated that tumour progression inhibits the inducibility of IFN in all the experimental systems used; alternatively, the generation of substances interfering with IFN action in the tumour-bearing hosts can be suggested, too.

POTENTIATION OF ANTITUMOUR ACTIVITY BY COMBINATION OF MURINE ALPHA, BETA AND GAMMA INTERFERONS

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Murine interferons were employed separately and in different combination in a mouse B16 melanoma tumour model. A statistically significant increased delay of tumour growth and survival time were seen in mice treated with various combinations of gamma interferon with either alpha or beta interferon. Combinations of these interferons gave an antitumour effect equivalent to a twofold to fourfold potentiation. Thirty percent of the mice

treated with the most potent combination of alpha and gamma interferon and forty percent of the mice treated with the most potent combination of beta and gamma interferon survived apparently tumour-free for 100 days. All control mice died by day 41. The study provides some promise for the utility of combination interferon therapy in the control of at least some tumours in man.

EFFECT OF INTERFERON ON THE ACTIVITY OF HUMAN AND CHICKEN GRANULOCYTES

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In chickens not the NK cells but the granulocytes reveal natural cytotoxicity. This cytotoxicity is enhanced by chick leukocyte-, fibroblast-, and immune interferons, whereas interferon-pretreatment of the target cells impaired their sensitivity to granulocyte-cytotoxicity. Cytotoxicity was estimated by ^{51}Cr release assay. Adenovirus stimulates cytotoxicity via interferon induction which can be neutralized by anti-chicken interferon antibody. In chicken not only the infection with adenovirus but the injection of interferon itself also led to a transient granulocytosis and stimulation of natural cytotoxicity. Human granulocytes do not exert spontaneous cytotoxicity even after pretreatment with human alpha or gamma interferon. At the same time, chemiluminescence of human granulocytes is more pronounced than that of chicken granulocytes, and can be stimulated by human gamma interferon and to a lesser extent by human alpha interferon. As viruses regularly induce interferon production, the modulation of granulocyte reactivity by interferons may be important in the defense mechanism against viruses in humans and birds as well.

RADIOPROTECTIVE EFFECT OF BETA INTERFERON IN MICE

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In previous experiments the effect of different kinds of irradiation on interferon induction in mice and the correlation between radiosensitivity of interferon production and the radioprotective activity of inducers was studied.

It has been reported that various interferon inducers of viral and synthetic origin act as radioprotectors when the "in vivo" induced interferon levels proved to be radioresistant. This fact indicates that interferons play a role in radioprotection. In the present experiments the radioprotective activity of exogenous murine beta-interferon irradiated with acute X-rays was studied in mice. Mice were irradiated with acute X-rays in doses of 4.8 Gy, 5.0 Gy and 5.2 Gy. Murine beta-interferon was administered intraperitoneally (1.8×10^3 – 1.8×10^4 IU/kg) 24 h before and 2 h after irradiation. Murine beta-interferon was induced in L-929 mouse fibroblast cultures by NDV (10 ELD₅₀/0.2 ml). The supernate was collected 24 h after incubation at 37 °C and treated with 60% perchloric acid. This crude preparation was assayed in L-cell cultures by the CPE inhibition test applying EMC virus as challenge. The titre of beta-interferon was 1.2×10^3 IU/ml, as compared to the international standard (WHO Mouse IFN, NIH-NIAID, USA). Administration of murine beta-interferon markedly increased the survival of irradiated mice: in dose 4.8 Gy from 55% to 80%, in 5.0 Gy from 30% to 90%, and even in 5.2 Gy, corresponding to LD₁₀₀, the survival reached 40%. These results confirm the role of interferon system in radioprotection.

EFFECTS OF INTERFERONS AND TUMOUR NECROSIS FACTOR ON DEVELOPMENT OF INTRACELLULAR BACTERIAL INFECTION

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Invasiveness of the epithelial cells in the gut by several facultatively intracellular enteropathogenic bacteria, as salmonellae, shigellae and enteroinvasive *Escherichia coli* is an important pathogenetic factor in the course of infection. This process is inhibited by pretreatment of cells with different types of interferons. It can be demonstrated in in vivo animal model and in cell culture models in vitro. Tumour necrosis factor seems to have minor effect on this process. It is mostly the internalization process that is reduced, but to a lesser degree also adhesiveness to the cell surface. The effect is dependent on active cell metabolism. Interferon effect was not expressed when cultures were treated with protein synthesis inhibitors. Also *Shigella dysenteriae* 1 toxin inhibited the anti-invasive effect and invasiveness of *Shigella* species in toxin-susceptible cells was not affected by interferon.

INHIBITED INTERFERON PRODUCTION AFTER SPACE FLIGHT

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Interferon production may be impaired after space flight. Using anti-orthostatic suspension of rodents we have shown that interferon-alpha/beta production is inhibited after suspension, and the inhibition is not solely due to the stress of suspension. Antiorthostatic suspension models some of the effects of weightlessness seen during space flight. The inhibition of interferon-alpha/beta was transient, in that suspended animals returned to normal caging for one week recovered the ability to produce interferon. Antiorthostatic suspension of mice also resulted in a loss of resistance of female mice to the diabetogenic effects of infection with encephalomyocarditis D virus. The loss of resistance correlated with the drop in interferon-alpha/beta production that occurs during suspension. In rats flown on the Space Shuttle in mission SL-3, interferon-gamma production was severely inhibited when spleen cells were challenged with concanavalin-A upon return to earth. In contrast, interleukin-3 production by rat cell was normal after space flight. These results suggest that immune responses may be altered after weightlessness modeling and space flight, and that resistance to viral infections may be especially affected.

STUDIES ON THE MECHANISM OF THE PRIMING EFFECT OF INTERFERON IN VIVO AND IN VITRO

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Injection of mice with murine interferon (IFN) alpha/beta before the induction of IFN by 3 μ g/ml poly rI : rC enhanced early IFN production. Peritoneal cells from primed and induced animals produced more IFN than cells from mice not primed before induction. The manifestation of priming was optimal when pretreating doses of 1500 to 3000 IU/g IFN were applied. Reduction of the amount of injected IFN below this level markedly decreased priming. The duration of IFN pretreatment also influenced establishment of the in vivo primed state, indicating a time- and dose-dependent induction of priming in vivo. The in vitro mechanism of priming was analyzed

in two lines of L929 cells, designated L929B and L929M. The L929B cells, which displayed a 2-3-fold higher IFN production in response to Sendai virus than the L929M cells, had a higher sensitivity to the antiviral and priming effects of IFN and were more resistant to VSV. IFN production and IFN-mRNA activities, quantitated through *Xenopus laevis* oocyte microinjections, were proportionally increased in the IFN primed cultures of both cell lines. The results indicate that the priming-induced increase of IFN production is based on a pretranslational control mechanism.

EFFECT OF INHIBITOR (ISIFN) ON INTERFERON SYNTHESIS IN DIFFERENT MOUSE CELLS

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Peritoneal macrophages produced small amounts of physiological interferon *in vitro*. Its synthesis is under control of natural inhibitor(s) and stimulator(s) which are released by macrophages. This inhibitor influenced the physiological as well as the LPS and NDV induced IFN production in peritoneal cells. The effect of the inhibitor in different cells, isolated from normal mice, was checked. On the contrary to IFN synthesis in peritoneal and bone marrow cells, which was sensitive to the inhibitor, the IFN synthesis in fibroblasts and peripheral blood leukocytes is insensitive to its action.

CONSTRUCTION OF TRANSGENIC MOUSE CARRYING EXTRA INTERFERON GENE

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As a means of elucidating physiological roles of interferon (IFN) in the body, we constructed transgenic mice carrying extra mouse IFN-beta gene, by microinjecting, into fertilized mouse eggs, the cDNA ligated downstream to the mouse metallothioneine-I promoter-enhancer region. So far, 14 mice were obtained, containing the exogenous mouse IFN-beta gene. The copy number of the exogenous gene varied from mouse to mouse; the injected DNA fragments were found in the chromosome ligated together in irregular arrays,

suggesting that the DNA fragments were ligated to each other through their cohesive ends and then integrated into the chromosome at one site. IFN activity was detected in sera of four transgenic mice out of 12 examined, in a range of 50–300 units per ml. Peculiarly, IFN- α was found to be present together with IFN- β . On the other hand, no IFN activity was detected in sera of five non-transgenic mice. IFN- β mRNA derived from the exogenous genes was detected in livers of two transgenic mice, in which IFN activity was detected. In conclusion, transgenic mice harbouring extra mouse IFN- β genes were obtained; in some of these mice, the exogenous IFN- β genes were expressed, but the endogenous IFN- α genes were also expressed. The reason for this is being explored.

MECHANISM OF INTERFERON INDUCTION

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The antiviral and antitumour properties of interferons (IFN) emphasize the importance of understanding how IFN production is controlled and how IFN genes are regulated. The signal for activation of IFN gene is the inducer. The effect of the inducer is probably transduced through transacting factors to the cis-regulatory elements of IFN gene promoter. By using the "gel retardation assay" we identified a factor interacting with the promoter of the human IFN- β gene. Experimental results suggest that this factor plays role in the regulation of the IFN gene. (i) The relative concentration of the factor is increased upon Sendai virus induction or poly I:C treatment. (ii) Changes in the factor concentration are parallel with the time course of the synthesis of IFN. (iii) Dissection of the promoter and footprint analysis localized the binding site of the factor to a region of the promoter, which is absolutely required for the regulated expression of the IFN gene. Competition experiments suggest that the IFN promoter specific factor interacts indirectly with the inducer molecule, probably through other trans-acting factor(s). Therefore, in the next step we investigated the presence of factor(s) binding directly to the inducer molecule. For this purpose a set of double-stranded RNA molecules were synthesized in vitro by SP-6 polymerase. The structural requirements for IFN induction were determined and factors probably involved in the signal transduction were identified by protein blotting procedure.

A SUPERINDUCTION-LIKE INCREASE OF IFN PRODUCTION BY BUTYRATE IN ADENOVIRUS INFECTED CHICK CELLS

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Treatment of human adenovirus-infected chick cells with arginin butyrate early after infection strongly inhibited interferon formation. No toxic effect or suppression of early viral function were observed. Treatment, however, at 24 h after infection causes a substantial increase in IFN yield. We conclude that butyrate treatment may have a relatively specific effect on the formation of inducer molecule or on cellular function necessary for induction. The enhancement of IFN expression late after infection can possibly be attributed to the same translation inhibitory effect of butyrate but this time it exerts a superinduction-like phenomenon preventing an IFN repressor mechanism. Support of this assumption was sought by inhibiting translation in adenovirus-infected chick cells different time after infection. Treatment of cells with cycloheximide resulted in preventing IFN formation early whereas considerable increase was observed late after infection. Thus the superinduction could also be demonstrated in the case of IFN induction by a DNS virus.

EFFECT OF EXTRACELLULAR SLIME FROM *PSEUDOMONAS AERUGINOSA* ON INTERFERON INDUCTION IN MICE

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The extracellular slime from highly mucoid strain of *Pseudomonas aeruginosa* 219 exhibited immunomodulating potency and was not toxic for mice and rabbits. Interferon was induced in sera and spleens of 129/AoBoy mice injected with the solution of slime (100 μ g/mouse). IFN appeared in sera 2 h after intravenous or intraperitoneal injection and was detectable till 24 h. In the spleens, interferon was observed from 2 h until the 7th day after injection of slime solution. Both interferons were produced by non-adherent cells. Peritoneal cells from mice pretreated with the slime solution, after second stimulation in vitro, synthesized higher amounts of IFN than

the control, nontreated ones. Comparison of the physicochemical and antigenic properties of IFNs present after induction in sera and that obtained after stimulation of peritoneal cells in vitro, showed that both of them were different.

LARGE SCALE PURIFICATION OF HUMAN, BOVINE AND PORCINE INTERFERON ALPHA AND GAMMA PREPARATIONS ON CONTROLLED PORE GLASS BEADS

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Controlled pore glass beads with pore size of 350–75 Å, equilibrated with PBS at pH 7.2 ± 0.2 adsorb efficiently alpha and gamma IFN molecules of human, bovine or porcine origin. After a thorough wash with 0.1 M Tris-HCl pH 7.4 ± 0.4 , all IFNs elute readily by 1–2 M NaCl buffered with 0.2–0.5 M Tris-HCl pH 8.2 ± 0.2 with a recovery of 96–99%, and with specific activities of $1-2 \times 10^7-10^5$ IU per mg protein (in the peak fractions). After desalting, subtypes of alpha IFNs were separated from CPG-purified preparations by chromatofocusing and their antiviral and anti-inflammatory effects were determined. Several IFN alpha preparations were compared, purified with different methods. Considering specific activity, yield and presence of subtypes in IFN alpha preparations, CPG-chromatography seems to be superior.

PROPERTIES OF INTERFERONS PRODUCED BY DIFFERENT CELL POPULATIONS

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Three interferon inducers — poly(G).poly(C), dsRNA phage Fz and dsRNA *Saccharomyces cerevisiae* — induced in mice interferon with different properties as far as pH and t-resistance concerned. “Early” serum interferon is different from “late” interferon not only at the level of production, but also reacts differently to the effects of the macrophage system, T-suppressor and T-helper cells. Peritoneal macrophages produce insignificant amounts of

interferon. The removal of adhesive fraction of monocytes does not effect the interferon production by spleen cells. At the same time, inhibition of the normal function of macrophage system by trypan blue leads to a substantial drop in the production of interferon by spleen cells independent of the type of the inducer used. Hydrocortisone changes the properties of the produced interferons, the hormone possesses the ability of inducing the synthesis of interferon by PBL and spleen cells. Varied interferons were produced by different cell populations of B- and T-lymphocytes and similarly by macrophages.

STUDIES ON THE EFFECTOR CELL POPULATION IN SENDAI VIRUS-INDUCED HUMAN LEUKOCYTES

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Interferon-(IFN)-producing cells belong in the mononuclear cell (MNC) fraction of human peripheral leukocytes. The granulocytes did not respond to Sendai virus induction. The plastic-adherent cells, comprising 12–15% of the total MNC, produced approximately two-thirds of the detectable IFN. The kinetics of primed and unprimed IFN production of the adherent, non-adherent and unseparated MNC were similar, as were the physicochemical and immunological characteristics of the IFN preparations produced by these cell fractions. In the presence of complement, MoAb specific for human monocytes inhibited only the IFN production of the adherent cells. Immunofluorescent staining with antisera to HuIFN alpha also demonstrated an evaluable number of fluorescence-positive cells only in this cell fraction. On a cellular basis, more poly(A)-containing RNA could be extracted from the plastic-adherent cells. This RNA fraction had a higher IFN-mRNA activity upon injection into *Xenopus laevis* oocytes than the RNA from the nonadherent cells. The cellular origin of the RNA preparations did not influence the immunological characteristics of the translated IFN.

ATTEMPTS TO CHANGE THE PHARMACOKINETIC BEHAVIOUR OF HuIFN-ALPHA

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Heterologous HuIFN-alpha displayed similar pharmacokinetics to that of homologous MuIFN-alpha/beta in mice. The main goal of the present experiments was to obtain HuIFN-alpha preparations which could persist longer in the circulation in comparison to the native HuIFN-alpha. Two methods were used. First, the HuIFN-alpha was complexed with blue-dextran (BD), and the conjugated and residual IFNs were separated by precipitation with polyethylene glycol (PEG). The use of 1% BD and 10% PEG was optimal for the preparation of these complexes. BD-bound IFN exhibited favourable pharmacokinetics in comparison to the native IFN injected either intramuscularly or intravenously into mice. In the second approach, HuIFN-alpha was treated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI), a homobifunctional crosslinking agent, in order to obtain bovine serum albumin-(BSA)-HuIFN-alpha complexes. Sephacryl S-200 gel filtration of these preparations revealed that 450 pm HuIFN-alpha and 67.5 nm EDCI were optimal for the recovery. Approximately 5.5% of the starting IFN could be detected in a biologically active form in the fractions corresponding to the molecular weight of BSA under these conditions.

BACTERIOLOGICAL FINDINGS IN URINE IN PATIENTS TREATED WITH HuIFN-ALPHA

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In the years 1978–1986, 68 patients with urinary bladder papillomatosis and secondary infection were treated with HuIFN-alpha. In addition to the usual clinical tests, bacteriological examinations were also performed. Crude interferon was given to all patients at 2×10^6 units daily. The administration was by transurethral route, using a surgical urethrocystoscope. The urine isolations showed *Escherichia coli*, *Klebsiella* and *Proteus mirabilis*. No adjuvant therapy with antibiotics was given in any phase of the trial. Within 10–15 days of the beginning of interferon therapy, bacteriological findings in the urine of all patients were negative. The results of the trial indicated an antibacterial activity of HuIFN-alpha.

ANTIVIRAL ACTIVITY OF DOUBLE STRANDED RIBONUCLEIC ACID AS INTERFERON INDUCER

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Interferon inducer — double stranded RNA (dsRNA) — was obtained from the biomass of *Escherichia coli*, infected with the ambermutant of bacteriophage f2. In vitro studies of the antiviral activity of dsRNA demonstrated a dose-dependent decrease of the test-virus yield to 4 log without establishing the detectable interferon titres. A combined use of dsRNA and interferon enhances the inhibitory effect. The most marked protective effect of dsRNA in vivo was obtained when interferon inducer was administered prior to infection. A similar phenomenon was observed when studying the effect of dsRNA on the development of infection in plants. A local application of a dsRNA-containing liniment in humans demonstrated a good therapeutic effect upon several viral infections of the skin and mucous membranes. The obtained data testify to the wide spectrum of the antiviral effect of dsRNA.

INTERFERON SYSTEM IN ARTHRITIS

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Forty arthritis patients were studied for interferon producing ability of peripheral blood leukocytes by the leukocyte IF reaction method of Solovyov. Mean geometric titre of IF alpha and beta changed 3.35 ± 0.21 and 2.50 ± 0.15 respectively in patients with active phase rheumatoid arthritis. Also, the relative and absolute number of B- and, especially, T-lymphocytes decreased; T-lymphocytes suppression activity was considerably reduced. Forty percent of the patients had no IF gamma titre, generally those who were subjected to longterm treatment with steroids. The IF-producing ability was normal in podagric and reactive arthritis. After the treatment which also included the immunocorrective drugs, i.e. leukocyte IF, T-activin, small doses of corticosteroids, the IF production somewhat increased; however, it did not reach the normal level. Disturbances found in the immune response regulation system require further study and corresponding treatment.

Symposium III

Latent and Persistent Viral Infections

COMPARATIVE STUDIES ON EXPERIMENTAL HERPES VIRUS LATENCY

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Two different models of herpesvirus persistence are described: that of human herpes simplex virus (HSV) in rabbits and mice and that of murine herpesvirus (MHV) — a new member of the family *Alphaherpesviridae* — in mice. In more than 100 rabbits and 50 mice kept for up to 380 days after corneal inoculation, the results of direct examination of the cornea, both Gasserian ganglia and brain stem were compared with those of explantation. The formation of HSV-specific antigens was followed using anti-virion serum and a serum to nonstructural polypeptides. Viral DNA sequences were searched for in cultured and noncultured tissues by two hybridization techniques. The results point at static latency in the ganglion in the absence of specific antigen formation. The positive hybridization in the brain stem was not in accordance with the explantation results. Persistence of MHV in the lung, spleen and kidney was of dynamic type being in sharp contrast with the static latency outlined above. Unlike to HSV, the MHV spread mainly by haematogenous route.

ASSOCIATION OF EPSTEIN-BARR VIRUS WITH MALIGNANT NON-HODGKIN'S LYMPHOMAS

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There is some experimental and clinical evidence that Epstein-Barr virus (EBV) has a causal relationship with lymphoproliferative disorders in patients with therapeutic or herited immunosuppression. Up to now, little is known about the association of EBV and malignant B-cell lymphomas in patients without a well-defined immunodeficiency. For that reason we investi-

gated 43 patients with malignant lymphomas of various entities for the presence of EBV DNA and EBV nuclear antigen (EBNA) in their tumours or lymph nodes, respectively. EBV DNA was detected by in situ hybridization in tissue specimens of 5 patients. EBNA could be demonstrated by anti-complement immunofluorescence in 3 of these patients. Histopathologic examinations using the classification of Lennert revealed that 2 patients had centroblastic lymphomas and each one of the remaining 3 had lymphocytic, lymphoplasmocytic and lymphoblastic lymphoma.

INVESTIGATION OF A PIG FARM LATENTLY INFECTED WITH AUJESZKY'S DISEASE VIRUS (PRV-1)

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In a farm 45.3% out of 450 breeding sows were positive for Aujeszky's virus antibodies tested in complement modified virus neutralization test. There was a close correlation between the age of sows and the rate of the infection (1.5 years, 23.3%; 2-2.5 years, 39.3%; 3-6 years, 73.3%). Only the sows played role in spreading of the infection. No virus was isolated from the nasal swabs collected from gilts and sows after mating and farrowing. No virus was cultured from swabs or from the gasserian ganglion of sero-positive (8) or seronegative (7) sows after immuno-suppressive treatment with 15 mg/kg glycocorticoid (Depersolon) on 4 consecutive days. By changing movement of pigs within the farm gilts, they could be kept free from Aujeszky's disease even after farrowing and weaning.

SUPPRESSION OF TRANSGLUTAMINASE LEVEL OF HEF CELLS INFECTED BY HUMAN CYTOMEGALOVIRUS

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The HCMV is implicated in the stimulation of macromolecular synthesis of infected cells, and the progression of normal cells to malignant tumour. There are sequences of HCMV-DNA specified for immortalization, oncogenic

transformation of normal cells and the DNA share homology with 5' coding sequences of viral and cellular myc oncogenes. The transglutaminase level was extremely low or undetectable in activated myc oncogene containing tumour cells. The transglutaminase level of normal cells significantly decreased, or was undetectable after productive and non-productive infection with HCMV. The significant suppression of transglutaminase level is associated with the HCMV early gene product(s). The degree of the inhibition of transglutaminase depends on virus multiplicity, cultural conditions and passage number of the cell culture. In this moment it is questionable whether transcriptional or translational control exists after HCMV infection associated with significant decrease of transglutaminase level.

HUMAN PAPILLOMAVIRUS INFECTION OF THE LOWER GENITAL TRACT

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Human papillomaviruses (HPV) seem to have an important role in the development of genital cancer. HPV-DNA replication occurs predominantly in the upper cell layers of the epithelium, therefore the sensitive in situ filter DNA hibridization method was satisfactory in stringent conditions (T_m -18 °C) to identify the different types of HPV DNA. Patients with normal cervical cytology and mild dysplasias were positive for HPV in 34-36%; JPV 11 occurred most frequently. Multiple HPV infection was detected in flat type condylomas (6 cases). In carcinoma in situ patients HPV 16/18 was identified (5 cases). Biopsies from cervical cancer were analyzed for HPV sequences by Southern blot technique, and HPV 16 DNA was identified.

Agricultural and Food Microbiology

INTERACTIONS BETWEEN PHYTOPATHOGENIC BACTERIA AND HIGHER PLANTS, EXEMPLIFIED BY *PSEUDOMONAS SYRINGAE* PV. *PHASEOLICOLA* AND BUSH BEAN (*PHASEOLUS VULGARIS*)

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Higher plants have developed a diversity of reactions against infecting microorganisms, which may occur successively or simultaneously: early induced resistance; late induced resistance; hypersensitive reaction (HR); synthesis of phytoalexins; release of agglutinins or lectin-like substances; warding-off reactions. These resistant reactions can be easily provoked in the plant after invasion of foreign organisms or by disturbance of metabolism. The fascinating phenomenon of plant diseases is, that although the plant tissue is heavily damaged by multiplying microorganisms, resistant reactions do not occur or are slowed down considerably. Especially, phytopathogenic pseudomonads and xanthomonads are characterized by a very narrow host range. Each pathovar can only infect one or very few closely related plant species. Thus, in their host plants, these bacteria inhibit the resistant reactions by very specific mechanisms. However, most of the virulence factors described from phytopathogenic bacteria are unspecific: e.g. toxins which cause chlorosis or necrosis; hydrolytic enzymes; the unknown principle which induces the hypersensitive reaction by damaging plasma membranes. Only for extracellular polysaccharides (EPS), which induce persistent watersoaking in the intercellular spaces, could be demonstrated a specific effect in some instances. The components of the EPS were analyzed as (i) levan (fructosan); (ii) alginate (mannuronan), correlated with virulence; (iii) lipopolysaccharides (LPS) and (iv) proteins, including levansucrase. From these, LPS or unknown proteins (which occur, however, in very low concentrations) may be the only compounds capable for specific interactions. Gene-technological studies, thus far, have only revealed virulence characters common for several *Pseudomonas syringae* pathovars.

MICROBIOLOGICAL CONTROL OF FOOD PROCESSING ON THE BASIS OF THE "HAZARD ANALYSIS, CRITICAL CONTROL POINTS (HACCP)" SYSTEM

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The microorganisms present in food products determine their keeping quality and safety, and have an effect on their sensory quality, nutritional value and convenience. The microbiological hazards in food processing depends on the presence of pathogenic and/or spoilage microorganisms which may cause illness or food spoilage losses. Microbiological critical points of the process, unless correctly controlled, may lead to contamination by microorganisms, or their survival or unacceptable growth. The 5-P means of the monitoring of food processing are: (i) plant construction, (ii) procurement of raw materials, (iii) personnel, (iv) processing for safety, and (v) preservation of microbiological quality and safety during transportation, storage and marketing. The development and application of the HACCP system is based on the knowledge of microbial ecology, food technology, chemistry, hygiene, biometrics and economy.

INFLUENCE OF TITAVIT R (TITANIUM CHELATE) ON PLANT LEAF PATHOGENS

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Titanium has a positive influence on the uptake of other minor elements and the production of some crops (quantity and quality) is positively influenced by titanium solution spray. Titavit R, a water soluble titanium chelate of the Hungarian firm Nitrokémia was tested on its activities against plant leaf fungi. Cucumbers were sprayed weekly with 2, 4 and 6 ppm titanium: although the total weight of the cucumber was quite the same, the infestation with *Sphaerotheca* (powdery mildew) was significantly decreased applying a 4 ppm spray. The influence of titanium on fungal growth in vitro (Petri dishes with PDA enriched with 0, 0.1, 1, 10, 100, 500 and 1000 ppm) was observed on *Alternaria* spp., *Phoma betae*, *Verticillium* spp., *Rhizoctonia solani*, *Fusarium oxysporum*, *Pythium*, *Penicillium* spp. For these fungi, there was

no influence of fungal growth in vitro. Experiments with beans sown in a soil heavily infested with *Rhizoctonia* showed no influence related to seed treatment and soil treatment, but spraying the young seedlings with Titavit R, significantly reduced *Rhizoctonia* infestation.

DECOMPOSITION OF EPTC IN SOIL BY MICROBES

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The decomposition of the herbicide EPTC (S-ethyl-*N,N*-dipropyl-thiocarbamate) was investigated in a soil with and without previous EPTC exposure. We isolated a bacterial strain from the soil previously exposed to EPTC twice, which could use EPTC as sole carbon and energy source. The decomposition of EPTC was investigated in the following samples: (a) sterile control; (b) no previous EPTC exposure; (c) previous exposure twice to EPTC; (d) sterile soil inoculated with EPTC-degrading strain (2.3×10^7). The samples were exposed to 50 mg.kg^{-1} EPTC and the EPTC content of soils was measured by gas chromatography. The degradation of EPTC was faster in all soils than in the sterile control. The EPTC content decreased to delation limit during 14 days in soil (b) while 5 and 7 days in soil (c) and (d). The decomposition of EPTC in soil samples depended on the activity of soil microbes, development of resistance to herbicides and previous EPTC exposure.

MICROBIOLOGICAL DECONTAMINATION OF LIQUID EGG PRODUCTS

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Liquid whole egg and egg-white were irradiated in order to reduce microbial cell count. Egg products were packed into polyethylene foil and frozen at -18°C . The samples were irradiated (^{60}Co dose range, 2–10 kGy; dosage rate, 10.9 kGy.h^{-1}). The samples were irradiated between cooling pouches thus their temperature rose only by $3\text{--}5^\circ\text{C}$. The decrease of microbiological contamination of non-pasteurized whole liquid egg and egg-white (in frozen state) was, in a high degree, a function of irradiation dose. The mesophilic aerobic microbial count was reduced by 3–4, *Escherichia coli*,

enterococci and *Staphylococcus aureus* by 3 exponents as an effect of 2 kGy. Salmonellae could not be demonstrated from the samples. Organoleptical and technological attributes of irradiated egg products satisfied the specifications required.

THE EFFECT OF HERBICIDES ON ANTAGONISTIC PROPERTIES AND THE SPECIAL COMPOSITION OF SOIL ACTINOMYCETES

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Actinomycetes were isolated from soils of the Taldy-Kurgan region in Kazakhstan. Sugar beet seedlings with them were treated with different herbicides (sabet, kerb, etsan, phenason, sodium trichloracetate, and a combination of the two latters). The incidence of actinomycetes-antagonists increased under the action of herbicides. By the amount of antagonists, phenason per se and phenason with sodium trichloracetate were the most effective, and etsan was the least effective. Rarely occurring actinomycetes forms were found. Streptomycetes of such series as *Achromogenes*, *Chrysomallus*, *Chromogenes*, *Fuscus*, *Albocoloratus*, *Helvolus*, *Ruber* and *Coerulescens*, prevailed. About 85.4% of actinomycetes isolated at the first stage of tests had an antibiotic activity. Five strains of actinomycetes inhibiting the growth of Gram-positive and Gram-negative bacteria to a great degree were revealed. They are of interest for further study as producers of new antibiotics.

EPIPHYTON YEAST MICROFLORA OF LUCERNE AND ITS EFFECT ON PHYTOPATHOGENIC *PSEUDOMONAS SYRINGAE* STRAINS

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The quantitative occurrence of epiphyton yeasts and other microbe-groups were studied and quantitative investigation of yeasts and filamentous fungi was made on the phyllosphere of lucerne and 8 other cultivated plants. The upper part of lucerne yeast strains of the *Cryptococcus*, *Sporobolomyces*

and *Rhodotorula* genera were predominant. The number of yeasts increased during the vegetation period, reaching $10^6 \cdot g^{-1}$ green plant at the end. Some yeast strains from the plants could inhibit the growth of *Pseudomonas syringae* strains in vitro.

COMPETITION OF *RHIZOBIUM* STRAINS IN SYMBIOSIS

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An experimental system was worked out to characterize *Rhizobium* strains for practical application of competition. Alfa-alfa seedlings were infected with mixtures of Nif⁻ mutants, and *Rhizobium* strains were studied in various cell-proportions and the efficiency of symbiotic nitrogen fixation was measured after 5–6 weeks by the acetylene reduction of nitrogenase. The more competitive the strains were, the smaller ratio in the mixture with the Nif⁻ mutant was satisfactory to produce highly active symbiosis. Experiments carried out with various genetically labelled strains, and identification of reextracted bacteria from nodules confirmed the results.

AGENTS OF RYE BACTERIAL DISEASES

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Rye was infected by *Pseudomonas syringae* pv. *atrofaciens* McCullogh, *Erwinia carotovora* pv. *carotovora* Jones, and also by saprophytic species *E. herbicola* Duggeli and *P. fluorescens* Migula. *P. syringae* pv. *atrofaciens* was the most wide spread and pathogenic. The strains *P. syringae* pv. *atrofaciens* were identical with isolates of this species from wheat and barley and also with *P. syringae* pv. *coronafaciens* from oats. Forms of weakly pathogenic species of *E. herbicola* and *P. fluorescens* induce rye infection only under favourable conditions.

APPLICATION OF LACTIC ACID BACTERIA IN MEAT INDUSTRY

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Lactic acid bacteria with adequate lactic acid producing capacity and catalase activity were isolated from the environment of the meat industry. A procedure elaborated for large scale production of these organisms is based upon the combined treatment of the polysaccharides of the nutrients in order to increase the assimilation value by ionizing radiation and by heat. This method is suitable to the production of starter cultures for the meat industry. Mono- and disaccharides were added to the meat pulp, inoculated with the starter culture to enhance the activity of the bacteria. It was possible to reduce the duration of the ripening period of the sausage from 5–12 weeks to 2–4 weeks.

MORPHOLOGY OF *BROCOTHRIX* *THERMOSPACTA* PHAGES

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Nineteen phages isolated from beef were stained with uranyl acetate and phosphotungstate and were examined in a Philips EM 300 electron microscope. Phages were tailed, had icosahedral heads, and belonged to three morphotypes. They were classified as *Myoviridae* (species A19) and *Siphoviridae* (species NF5 and BL3). Type A19 included 4 Canadian isolates. Heads were of 90 nm in diameter and tails measured 170×17 nm, showing necks, collars, base plates, and spikes. Type NF5 comprised 14 isolates from Canada or France. Heads measured 58 nm in diameter. Tails were rigid and 105 nm long, terminating in numerous short fibers. Type BL3 was represented by a single isolate from France. Particles had 61 nm wide heads and flexible, about 260 nm long tails. Type A19 had a close resemblance to well-known phages of *Bacillus*, *Staphylococcus* and *Streptococcus* (species SP50, Twort, RZh), indicating phylogenetic relationships. The taxonomic position of *Brocothrix* may need revision.

EFFECT OF CARBAMIDE-TYPE HERBICIDES ON PURE CULTURES OF SOIL MICROORGANISMS

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Effects of four herbicides of identical basic structure but of different substituents have been tested on pure cultures of soil-borne fungi and bacteria under laboratory conditions. These agents were Dicuran, Patoran, Maloran and Afalon. Test methods consisted of measuring diameters of inhibiting, weakening, stimulating zones on meat extract and soil extract agar media. Evaluation was based on the slope of the straight line obtained by linearizing the concentration-diameter relationship $y = (ax + b)$ per x . This slope is proportional to the effectivity of the agent. The inhibiting effect on bacilli has been stated to decrease in the following order: Dicuran, Patoran, Maloran, Afalon. The effect reduces in the inverse order for *Pseudomonas fluorescens*. *Azotobacter croococcum* is only weakened, at the same time the capsulation time is reduced. After having determined the mass of fungi cultivated on malt juice in agitated flasks, the result is indicated as a percentage of the control specimen. The effectivity of inhibitive effect is reduced in the following order: Maloran, Afalon, Dicuran, Patoran. The most sensitive to react was *Mucor*, while *Trichosporon viride* exhibited the highest resistance.

PLANT INOCULATION WITH PGPR SIDEROPHORE PSEUDOMONADS

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The growth stimulative and amensalistic properties of the most effective strains of 80 rhizobacterium (PGPR) strains isolated from the rhizosphere and rhizoplane of different plants were investigated on apple seedlings in three soil types. As an effect of inoculation with single strains or strain combination, 22% plant growth surplus was observed in a slightly acidic brown forest soil. The inoculation and fungicide treatment (Thiram, Captan, Dithane) applied together produced even more favourable results. The effectiveness of inoculation was markedly influenced by ecological factors.

ANTIBIOTIC SENSITIVITY OF *RHIZOBIUM LEGUMINOSARUM* BV. *VICIAE* STRAINS

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The sensitivity of different strains of *Rhizobium leguminosarum* bv. *viciae*, *Rhizobium leguminosarum* bv. *phaseoli* to 34 antibacterial agents was measured using agar diffusion method. *R. leguminosarum* bv. *viciae* streptomycin mutant was the least sensitive to these agents, while *R. leguminosarum* bv. *phaseoli* was the most sensitive. All strains were sensitive to chlortetracycline, polymyxin-B, azlocillin, carbenicillin, amikacin, neomycin, oxytetracycline, ampicillin, chloramphenicol, gentamicin and tetracycline. The most marked inhibitory effect was shown with tetracycline at 50 mg.l⁻¹. The strains for inocula can be selected on the basis of their tolerance.

EXTENDING SHELF LIFE OF SWEET CORN BY SHRINK-WRAPPING, REFRIGERATION AND IRRADIATION

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Chemical, physical, organoleptic and microbiological changes were monitored during refrigerated (10 °C) storage of shrink-wrapped fresh sweet corn on the cob. Post-harvest deterioration was observed in terms of decreased glucose and soluble solids concentration, increased starch and alcohol insoluble solids content, decreased pH, and a darkening in colour and increasing in chewiness with storage time. These changes were significantly retarded by shrink-wrapping that essentially eliminated moisture loss and resulted in elevated (5–10%) carbon dioxide and decreased (10–15%) oxygen concentrations within packages. These effects, together with refrigeration, resulted in at least a three-fold extension in shelf life. The humid atmosphere, however, enhanced the microbial growth on shrink-wrapped corn. The aerobic microbial population were at least one log₁₀ unit higher than in unwrapped controls. Storage temperature changed the composition of the microflora. At 10 °C, the pseudomonads, micrococci and basidiomycetous yeasts contributed to a higher percentage of the microflora than at 20 °C, the enterobacteria, enterococci and fermentative yeasts were restricted on corn stored at 10 °C. The initial microbial population was decreased by 2–3 log₁₀ and by 0.5 and 1.0 kGy (⁶⁰Co) irradiation.

DETECTION OF *AGROBACTERIUM* *TUMEFACIENS* BY ELISA

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A double antibody sandwich method of the enzyme-linked immunosorbent assay (ELISA) was developed for the detection of *Agrobacterium radiobacter* var. *tumefaciens*. Antisera were produced by the immunization of rabbits with virulent and non-virulent bacterial strains. The DEAE-purified IgG were conjugated with horseradish peroxidase by the periodate method. The sensitivity of ELISA test was about 10 000 cells/ml. The method was used to determine the bacterial strains isolated from different sources: grapevine, apple, plum, cherry, peach, raspberry, rose and chrysanthemum plants from Moldavia.

THE EFFECT OF MICROWAVE TREATMENT ON *ESCHERICHIA COLI*

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The lethal effect of 2.45 GHz microwave irradiation was modeled on *Escherichia coli* test-system. The suspensions were equilibrated in pure nitrogen and air. The Specific Absorption Rate (SAR) was determined with temperature gradient measurement by thermocouple. SAR was 204.2 mW/g in the microwave-heated system and 100 mW/g in the thermostated system. The survival of the N₂ equilibrated suspension was higher by 53% than the air equilibrated one, as the effect of an average increasing in temperature by 36 °C. The surviving was also higher by 46% than the heat-treated suspension in N₂ without irradiation. In a thermostated system the thermal effect of microwave was eliminated. The survival of *E. coli* in N₂ remained on the control level but the survival of the air equilibrated suspension decreased nearly by one order of magnitude as an effect of one hour microwave treatment.

COMPUTERIZED EVALUATION OF FOOD MICROBIOLOGICAL CONTROL DATA

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In every year 300–500 data for each plant products are evaluated. The microbial counts of food samples show a log normal distribution in 90% of the products. The significance of the distribution-curve calculation is as follow: (i) to establish the adequacy of microbial counts of food to the limits of food hygienic standards; (ii) the annual microbiological quality of foods (by their parameters of mean values and standard deviations) may be compared and the trends of the changes can be seen; (iii) the endproduct specifications of spoilage organisms can be determined by taking the stability tests into consideration; (iv) the above points of view can be applied to the development of the methods of good manufacturing practice.

ANTAGONISTIC MICROBES ON WHEAT AND RED CLOVER SEEDS

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From the surface of wheat (Mv 8 cultivar) and red clover (Fertődi M cultivar), respectively, 50 and 34 microbial strains were isolated on nutrient, King-B and malt or casein containing media. The number of microorganisms was 10^5 – 10^6 on wheat seeds and 10^2 – 10^3 on red clover seeds. Most bacterial strains on red clover belonged to the genus *Bacillus*; among the strains on wheat seed there were no sporeforming bacteria. From the wheat seeds *Aspergillus*, *Penicillium*, *Acremonium* and *Mycelia sterilia* filamentous fungous strain representatives were isolated, from red clover seeds *Aspergillus versicolor* and other *Aspergillus* strains. Some of the microbial strains proved to be antagonistic to the other spermospheric microbes.

SIDE-EFFECT OF DIFFERENT PESTICIDES ON *RHIZOBIUM LEGUMINOSARUM*

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The side-effect of 23 pesticides on different strains of *Rhizobium leguminosarum* bv. *viciae* was compared with the same effect on the other biovars (*trifolii* and *phaseoli*) of that species and on a strain of *Rhizobium loti* in liquid culture using microfermentor and biophotometer techniques. The strain "Lóbab Z" *R. leguminosarum* bv. *viciae* proved to be the most tolerant and a strain of *R. leguminosarum* bv. *trifolii* was the least tolerant to the tested pesticides. Among the pesticides, the fungicide Orthocide was the most inhibitory and Acetochlor herbicide was the least inhibitory agent.

DOUBLE AND SINGLE STRANDED RNA OF PEA ENATION MOSAIC VIRUS

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The pea enation mosaic virus is an economically important virus in pea and broad bean in Hungary. Ds-RNA was purified from phenol-treated STE buffered extracts from leaf tissue, two cycles of fractionations on column Whatman CF-11 cellulose in the presence of 16% ethanol. Samples were treated with T₁ RNase and DNase after elution from the first column. Beside of the RF and RI ds-RNAs of PEMV, one out of three isolates investigated contained a satellite-like small RNA (estimated mol wt 2.2×10^5), which was characteristic of this isolate independently from host, mechanical and vector transmission. As proved its extraction from purified virus, the satellite-like RNA is encapsidated by virus protein. Strain of PEMV that carried this RNA produces much more severe symptoms on pea and broad bean than the other two strains.

THE ROLE OF PARASEXUAL RECOMBINATION IN THE EVOLUTION OF *FUSARIUM OXYSPORUM* FORMAE AND RACES

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According to the earlier general opinion, formae and races of *Fusarium oxysporum* specialized to different plant species and cultivars, respectively, are genetically isolated and the new formae and races are mainly arising as a result of mutation. Auxotrophic mutants of formae and races pathogenic to tomato, cabbage and *Gladiolus* were hybridized by means of protoplast fusion and hyphal anastomosis. The different formae and races were found easy to hybridize if the markers were favourably chosen. Stable hybrids were frequently obtained by means of both protoplast fusion and anastomosis, even inter formae, if the complementary auxotrophic markers were in different linkage groups. If the markers were on the same chromosome, the hybrids were formed as a result of mitotic crossing-over; this kind of recombination was of very low frequency, even within strains and forced by protoplast fusion. The ability of *F. oxysporum* formae and races to hybridize depended rather on the genetic markers used than on the genetical distance. From this we conclude, that formae and races of this important polyphagous fungus species arise in nature through parasexual recombination.

R-FORM OF *PSEUDOMONAS SYRINGAE* PV. *PHASEOLICOLA*

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The wide distribution in nature of virulent R-form of *Pseudomonas syringae* pv. *phaseolicola* has been observed. At transfer from S-form to rough form, the agent retains virulence, exhibits different colony morphology, decreases fermentative activity, changes the relation to phages and antibiotics. It does not react in S-antisera, decrease the rhamnose (7 times), glucosamine and galactose (2 times) content of lipopolysaccharide. Since rhamnose is the dominating monosugar of LPS-S, it may have a specific role in immunological specificity of bacterial cell and relation to phages.

THE EFFECT OF WATER-FREE AMMONIA ON BIOLOGICAL ACTIVITY OF THE SOUTHERN SOILS IN UKRAINE

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Ammonia was introduced into soil in large amounts (150, 300, 500 kg/ha). Laboratory and field tests showed that ammonia in the amount of 150 kg/ha and more, exerted negative influence on the functioning microbial community of soil, greatly changing its structure. The humus content tends to decrease as a result of introducing large doses of ammonia into soil.

ANTIGENIC COMPOSITION OF *PSEUDOMONAS SYRINGAE* PV. *PISI* SEROGROUP IV ON THE BASIS OF LIPOPOLYSACCHARIDE STRUCTURE

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When using various serological reactions and O, OH and absorbed antisera, *Pseudomonas syringae* pv. *pisi* was shown to contain the main factors "a, b, d". The other members of serogroup IV of phytopathogenic pseudomonads were characterized by factors "a, b, c". Methylation data, Smith's degradation, ^1H and ^{13}C NMR-spectroscopy showed that the main chain of specific polysaccharide of *P. syringae* pv. *pisi* was presented by tetra-D-rhamnan with side substitution of beta-D-glucosamine. Structural differences in the O-specific polysaccharide of LPS were not detected.

LIPOPOLYSACCHARIDE OF *PSEUDOMONAS SYRINGAE* PV. *PHASEOLICOLA*

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LPS was extracted with 0.85% NaCl and purified by ultracentrifugation. The serologically active preparation contained 37.5% carbohydrates and about 3% protein. Rhamnose, fucose, glucosamine and galactosamine in the

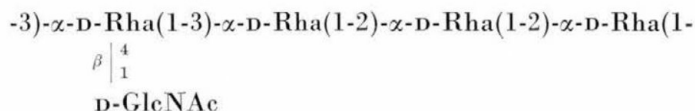
LPS were detected with paper and gas liquid chromatography. On mild acid degradation of LPS, an O-specific polysaccharide active in various serological tests was obtained. With the methylation analysis and Smith degradation, ^1H and ^{13}C NMR spectroscopy data the structure of LPS was determined. The O-chain of LPS in *P. syringae* pv. *phaseolicola* is a pentasaccharide containing D-rhamnan as a basic structure and one residue of D-fucose as a branching substituent.

LIPOPOLYSACCHARIDE OF *PSEUDOMONAS SYRINGAE* PV. *GLYCINEA*

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From the wet cells of *Pseudomonas syringae* pv. *glycinea* the LPS was extracted with 0.85% NaCl. In LPS, as main components of the polysaccharide part, D-rhamnose and N-acetyl-D-glucosamine were detected in ratio 4:1. Basing on ^1H and ^{13}C NMR spectroscopy, methylation and Smith degradation analysis, etc. the repeating unit of O-chain is:



This structure is characteristic of numerous strains of different pathovars of *P. syringae*.

LIPOPOLYSACCHARIDES OF *ERWINIA CAROTOVORA* SUBSP. *CAROTOVORA*

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In *Erwinia carotovora* subsp. *carotovora* LPS galactose, glucose, arabinose, heptose and aminosugar (glucosamine) have been found. The preparations differ in the presence or absence of mannose, xylose, fructose and rhamnose. Dodecanoic and oxytetradecanoic acids are the predominating fatty acids of lipid A. During cultivation all strains of *E. carotovora* subsp. *carotovora* produce a carbohydrate-containing biopolymer of galactane type.

FATTY ACID COMPOSITION OF PECTOBACTERIA

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Erwinia carotovora and *Erwinia chrysanthemi* differ from one another and from other members of the genus and family by qualitative composition and quantitative content of fatty acids of common lipids, the main components being hexadecanoic, hexadecenoic and octadecenoic acids. The absence of cyclopropane fatty acids, high content of unsaturated fatty acids, and weak synthesis of fatty acids with 17 carbon atoms are characteristic. *E. carotovora* has a low content of tetradecanoic and an increased content of dodecanoic acids. *E. chrysanthemi*, except *E. carotovora* f. sp. *zeae* and *E. chrysanthemi* pv. *paradisiaca*, does not or weakly synthesize dodecanoic acid.

THE OCCURRENCE OF AN *ACTINOPLANES* SP.
IN CHERNOZEM-BROWN FOREST SOIL

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We have found an *Actinoplanes* sp. in the A-horizon of a chernozem-brown forest soil located in the area of Visonta (East Hungary). In cultural and physiological properties 30 strains were relatively stable, but they proved to be hardly identifiable taxonomically due to their extreme variability concerning the structure of sporangia. The taxonomic differentiation of *Actinoplanes* is, at present, based chiefly on their sporangial morphology. But our findings clearly show that this approximation to the taxonomy of such sporangia-producing actinomycetes can lead to confusion. More stable physiological and biochemical features will be necessary for a reliable differentiation of *Actinoplanes*.

EFFECT OF BIOPREPARATIONS ON THE FUNGAL MICROFLORA CHANGES IN THE SUGAR BEET RHIZOSPHERE

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To protect sugar beet roots from rot pathogens in field conditions, biopreparations were tested. Before seeding, sugar beet seeds were treated with the polyene antibiotic roseofungin, obtained from *Streptomyces roseoflavus* strain A-23/791. Trichodermin (*Trichoderma lignorum* strain T-889) was introduced into soil at a dose of 0.5–3.0 g/m². Phytotoxic and phytopathogenic micromycetes were isolated from soil samples collected during the vegetation period. Antagonists to root rot pathogens were found among fungi. Phytotoxic and phytopathogenic fungi (43.3%) were shown to be wide-spread in the rhizosphere of sugar beet. When they were treated with biopreparations, their incidence decreased to 21.2%. During mass seedlings, about 40.3% of the fungi isolated were antagonists to phytopathogens, and by end of the vegetation period their number decreased to 18.7%. The majority of antagonists belong to the following genera: *Aspergillus*, *Fusarium*, *Penicillium*, *Aphanomyces*.

BIOLOGICALLY ACTIVE SUBSTANCES FROM FUNGI OF THE SUGAR BEET RHIZOSPHERE

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A search for antagonists against phytopathogenic fungi of the sugar beet rhizosphere was performed in the south of Kazakhstan at different crop rotations using the preparation trichodermin T-889. Of a total of 820 tested strains belonging to different fungal genera, *Penicillium lanosum* 947 and *Aspergillus terreus* 1987 were isolated. These cultures had a high activity (729 µg/ml) against sugar beet root rot pathogens. An antibiotic with polyene structure was isolated from *A. terreus* 1987 by thin-layer chromatography. The antibiotic inhibited the growth of *Fusarium oxysporum*, *Rhizoctonia violacea*, *Alternaria tenuis* at 30 µg/ml. *P. lanosum* 947 produced alkaloid lanosine which had activity against *Rhizoctonia adercholdii*, *Rhizoctonia solani*,

Botryomyces cinerea, *Fusarium solani*, *Fusarium oxysporum*, *Erwinia carotovora*, *Bacillus mycoides* at a concentration of 1.5–5 $\mu\text{g/ml}$. Tests of alkaloid lanosine and the polyene antibiotic against sugar beet root rot pathogens showed a decrease in the disease by 20–25% under field conditions.

THE INFLUENCE OF NITROGEN AND PHOSPHORUS NUTRITION SOURCES ON SYNTHESIS OF PHYTOTOXIC SUBSTANCES IN *MYCROCYSTIS AERUGINOSA* KÜTZ. EMEND. ELENK

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Mycrocystis aeruginosa, one of the main agents of the bloom of water reservoirs in the Dnieper, produced phytotoxic substances of high biological activities. *M. aeruginosa* has high phytotoxycity on Fitzgerald 11 medium which was taken as initial one for study of the influence of the various nitrogen and phosphorus components on the formation of phytotoxic substances. The highest phytotoxicity of cyanobacteria was found in the presence of KNO_3 and $(\text{NH}_4)_2\text{HPO}_4$. All phosphorus sources examined increased the growth and toxin formation. Rather high activity was shown with NaH_2PO_4 and $(\text{NH}_4)_2\text{HPO}_4$. As source of nitrogen and phosphorus, $(\text{NH}_4)_2\text{HPO}_4$ exerted positive effect on the synthesis of phytotoxic substance.

RAPID YEAST CELL COUNTING METHOD IN FRUIT JUICES AND WINE WITH LABORS-SCALE INSTRUMENT

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A rapid yeast cell counting method has been developed using particle counter instruments Picoscale and Laborscale (Medicor, Budapest). The parameters of measurement were as follows: diameter of capillary, 70 μm , and intensity of current, 400 μA . The yeast cell count of different orange and currant juices and wine was determined by particle counter compared to the

reference plate count method using OGY medium and 3 days incubation time. Good correlation was found between the two methods, the correlation coefficients being in the range of 0.88–0.99. The method is simple, it takes 20–40 s; the lowest limit of detection is 1000 cells/ml, compared to the 100 000 cells/ml limit of the direct microscopic counting method.

APPLICATION OF PICOSCALE PS-4 PARTICLE COUNTER IN THE MICROBIAL CONTROL OF APPLE CONCENTRATES

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The Picoscale PS-4 instrument used for the counting of corpuscular elements of the blood was studied for microbial control of samples taken at critical points of apple concentrate production. The yeast count determined by traditional plate count method was compared to the particle number measured by the instrument. A close correlation and a linear functional relation has been found between the results above a certain particle number ($r > 0.99$). The quality not complying with the GMP (Good Manufacturing Practice) can be detected within some minutes giving an opportunity to the operator to a rapid action.

LYSOGENY OF *XANTHOMONAS CAMPESTRIS*

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When studying the effect of UV-irradiation and mytomycin C on bacteria, lysogeny of *Xanthomonas campestris* was revealed only after UV-irradiation. All 37 examined variants and 7 strains of various *X. campestris* pathovars were lysogenic. Using strains of *X. campestris* pv. *cucurbitae* as indicators, the lysogenic variants were found in 64.8%. Twenty-two temperate phages were isolated: 14 from variants and 8 from pathovars of *X. campestris*. The isolation of temperate phages from variants of *X. campestris* pv. *campestris* was possible only in experiments conducted in the second part of summer. In winter and spring months no positive results were obtained. The isolated temperate phages can be used for study of bacterial variability of the genus *Xanthomonas*.

PATHOGENICITY OF *PSEUDOMONAS* *AERUGINOSA*

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Phytopathogenic characteristics of 26 clinical strains of *Pseudomonas aeruginosa* belonging to various serological groups were examined. In artificial infection the strains induced nodulated and warm thickness on bean stems and soft and wet rot of potato, carrot and onion slices. They did not induce typical hypersensitive reaction, but only chlorosis on tobacco leaves.

Bacteriology and Parasitology

THE FREQUENCY OF AEROBACTIN PRODUCTION AND ITS CORRELATION WITH PATHOGENITY IN HUMAN *ESCHERICHIA COLI* STRAINS

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Out of 632 human *Escherichia coli* strains tested by biological assay, 349 (55%) were aerobactin producers. The highest frequency was found among strains isolated from blood (80%) and CSF (84%) and among strains belonging to serogroups O2 (81%) and O18a, c (87%). There was a good correlation between LD₅₀ values (in intraperitoneally infected mice) and the frequency of aerobactin production. Aerobactin production was more frequent than the average among strains carrying single virulence factors as K1, K5, MRHA (mannose-resistant haemagglutination) and col V or their combinations. Only the haemolysin (HLY) positive strains showed a lower association than the average, but in combination with the above-mentioned factors the occurrence of the aerobactin production was higher (e.g. strains of K5+MRHA+HLY were aerobactin positive in 96%). Although the frequency of aerobactin production depends on selective conditions varying with the origin of the strains, the role of clonal relations have to be also considered.

PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF MULTIRESTANT *PROTEUS MIRABILIS* ISOLATES

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According to a survey on tendencies of antibiotic resistance in Hungary (1974–1983), the emergence of multiresistant *Proteus mirabilis* isolates is new phenomenon. A total of 60 strains resistant to 10–16 antibiotics were collected from various specimens and geographical locations. Third generation cephalosporins except for cefoperazone, amikacin, netilmicin and ofloxacin were effective in vitro against multiresistant *P. mirabilis* isolates. Out of them 62% belonged to O18, a serogroup not shown in early studies in Hungary;

moreover, in serogroup-distribution the present collection of strains differed sharply from isolates examined in 1956. All but two strains produced the same aminoglycoside-modifying enzyme ANT (2''), and all strains proved beta-lactamase positive in nitrocephin test. All multiresistant strains harboured 2-4 plasmids, whereas the sensitive isolates had no plasmids. The presence of two nontransferable plasmids (62 and 22 Mdal) occurring in 86.7% of the isolates was a characteristic feature of the multiresistant *P. mirabilis* strains. Some other plasmids (17.5, 103 and 113 Mdal) could be transferred to *Escherichia coli* recipient. There was no relationship between plasmid profiles, serogroups and resistance patterns.

HAEMOPHILUS INFLUENZAE AS A POSSIBLE RESERVOIR OF RESISTANCE PLASMIDS IN THE UROGENITAL TRACT

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Two taxonomically different *Haemophilus* strains isolated from a male patient with urethritis showed the same antibiotic sensitivity pattern: they were resistant to ampicillin, chloramphenicol, tetracycline and co-trimoxazole. *H. influenzae* strain HI 225 harboured 5 extrachromosomal DNA elements of 2.3, 3.1, 3.9, 4.4 and 24.5 Mdal. The beta-lactamase production and the sulphonamide resistance proved to be conjugative into *Neisseria lactamica*, *N. flavescens*, *N. meningitidis*, but not into *Escherichia coli* recipients. These experiments suggests the important role of *H. influenzae* in disseminating resistance factors among commensal and pathogenic bacteria.

ACTUAL ANTIBIOTIC RESISTANCE OF INTESTINAL BACTEROIDES IN HUNGARY AND THE GDR

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Intestinal *Bacteroides* of different species isolated recently in Hungary and the GDR from patients with anaerobic infections were tested by agar dilution method to clindamycin, lincomycin, erythromycin, oxytetracycline,

chloramphenicol, azlocillin, mezlocillin, cefoxitin, and metronidazole. Clindamycin, chloramphenicol and metronidazole exerted the best activity. Therefore, infections by intestinal *Bacteriodes* requiring a presumptive chemotherapy should be treated with one of these three substances. Considering that most anaerobic infections are mixed infections with aerobic or facultative anaerobic bacteria the latter must be included in the presumptive chemotherapy.

SUSCEPTIBILITY OF COAGULASE-POSITIVE STAPHYLOCOCCI TO STAPHYLOCOCCIN A

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An in vitro study of the susceptibility of 164 epidemiologically unrelated coagulase-positive *Staphylococcus* strains to staphylococcin A (STA) was performed by the microdilution assay. Susceptibility of 97 *S. aureus* strains (42 of biovar A, 23 of biovar B and 32 of biovar C), 37 *S. hyicus* and 30 *S. intermedius* strains was tested. At concentrations of 64 units/ml or less (a break-point for distinguishing susceptible and resistant strains) STA inhibited 62.2% of *S. hyicus* strains, 50% of *S. intermedius* and only 33.0% of *S. aureus* strains. Within each biovar of *S. aureus* the MIC distribution was significantly different from that of the whole strain population.

CLINICAL ROLE OF POTENTIALLY PATHOGENIC STAPHYLOCOCCI

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On investigating the clinical importance, antibiotic susceptibility and virulence of coagulase-negative staphylococci, 609 coagulase-negative staphylococcal strains were isolated in 5 years from clinical samples. As a whole, *Staphylococcus epidermidis* (42.5%), whereas in purulent cases the haemolytic species (*S. cohnii*, *S. epidermidis*, *S. haemolyticus*, *S. simulans*, *S. warneri*) were predominant (78.9%). The isolates proved to be more resistant as indicated by literary data. The incidence of multiply resistant strains was high (63.0%), only vancomycin was effective on all strains. Extracellular slime factor, an important virulence marker in foreign body infections was produced by 41.1% of the isolates. This phenotype is 2 to 3.5-fold more frequent in clinically significant strains than in contaminants.

THE EFFECT OF BETA-LACTAM ANTIBIOTICS ON CELL SURFACE HYDROPHOBICITY OF CONGENIC METHICILLIN-RESISTANT AND -SUSCEPTIBLE *STAPHYLOCOCCUS AUREUS* STRAINS

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A comparison was made on the degree of cell surface hydrophobicity (CSH) in 9 oncogenic methicillin-resistant (MR) and methicillin-sensitive (MS) pairs of *Staphylococcus aureus* strains. Cultivations were made on horse blood agar at 37 °C for 18 h. CSH was measured parallel with the improved salt aggregation test (SAT) and with hydrophobic interaction chromatography (HIC) using Octyl Sepharose CL B4 gel. The results of both the SAT and the HIC were in accordance showing methicillin resistance and CSH to be independent from each other at the level of a given MR-MS pair. On the whole, MR cocci possessed higher CSH than did MS ones. This might be advantageous for MR cocci in adhesion. The effect of various concentrations of methicillin and cefamandole on CSH of 2 MR-MS pairs was also studied during growth in casitone-glucose broth. Under the influence of beta-lactam antibiotics the degree of CSH in both the MR cocci and the MS cocci became higher measured with either the SAT or HIC. The changes were dependent on the antibiotic concentrations. The appearance of the aggregation forms in the SAT of both types of cocci also changed from granular to filamentous.

DISTRIBUTION AND ANTIBIOTICS RESISTANCE OF COAGULASE-NEGATIVE STAPHYLOCOCCI (CNS) ISOLATED FROM HUMAN INFECTIONS

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The species distribution of 364 CNS isolates was studied by a computerised program. There was an absolute dominance of *Staphylococcus epidermidis* (45%), isolates of an unclassifiable "interspecies" group occurred in 21%. The frequency of *Staphylococcus haemolyticus* was 17%. There was a significant difference in the frequency of CNS isolates studied according to

diseases and clinical specimens; 20–30% of the isolates were obtained from blood and cerebrospinal fluid. In $\frac{1}{3}$ of the cases the clinical significance of these findings could not be estimated on the basis of available information. Strains of *S. haemolyticus* were the most resistant against commonly used antibacterial agents, exhibiting 54–59% simultaneous resistance to methicillin, oxacillin and cephalixin determined by the disk diffusion method. This frequency was twice as high as that for *S. epidermidis*. A partial dissociation of cross-resistance to oxacillin (24%) and methicillin-cephalexin (32%) was revealed in isolates of the “interspecies” group. There appeared a 51% cross-resistance to erythromycin and oleandomycin in *S. epidermidis* and 64% in *S. haemolyticus* isolates. The isolates were resistant to oxytetracycline in 82–96% and to trimethoprim + sulfamethoxazole in 67–80%. The results indicated that members of the normal flora can easily become resistant against commonly used antibacterial agents.

STAPHYLOCCAL INFECTIONS IN DAIRY FARMS

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Milk samples from mastitis yielded staphylococci in the years 1984, 1985 and 1986 in 79.5%, 68.6% and 75.7%, respectively. In two years 840 strains were isolated from 792 cows in 47 dairy farms of Hajdú-Bihar county: 413 *S. aureus* and 427 coagulase-negative staphylococcus. *S. aureus* caused the most considerable latent infections as well as mastitis epidemics. In several dairy farms *S. chromogenes* and *S. simulans* caused milder infections. Of the cases 6.1% were reinfections.

NEW MYCOPLASMAS OF GOOSE ORIGIN

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In the last four years numerous *Mycoplasma* strains have been isolated from goose flocks affected by inflammation of the phallus. The majority of these isolates differs from the species described previously. Six distinct strains not corresponding to the known *Mycoplasma* species were found. Three of these (a glucose-splitting and two arginine-positive strains) have been reported

previously. One of the arginine-positive strains proved to be identical with *Mycoplasma cloacale* described in 1984. The incidence of these organisms as determined by indirect epifluorescence was: "1220", 345; "1219" 37; *M. cloacale*, 148. From the 29 negative samples further three strains were separated. By biochemical and serological tests these strains were not identifiable with the species described. One of them belonged to the *Acholeplasmataceae* family, while the other two to the *Mycoplasmataceae* family. A similar situation was found in the neighbouring countries where attempts at identifying the isolates failed. We have found these foreign isolates identical with the Hungarian ones.

POSSIBLE ROLE OF *MYCOPLASMA ANATIS* IN A DUCK DISEASE ASSOCIATED WITH SYMPTOMS OF THE CENTRAL NERVOUS SYSTEM

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A disease associated with symptoms of the central nervous system was observed in two duck flocks, each consisting of 6000 three-week-old ducklings. The symptoms were: weakness of legs, dyspnoe, diarrhoea and in many cases disturbance of the equilibrium, reverse moving, torticollis and necktwist. The pathological and histo-pathological examinations revealed lympho-histiocytic meningitis and cerebroventriculitis, airsacculitis with discrete fibrinous exudate, bronchitis associated with formation of lymphoid follicles, focal, interstitial pneumonia, in some cases with fibrinous serositis, interstitial catarrh and splenomegaly. Large numbers of mycoplasmas could be detected by electron microscopy and isolated from the affected meninges and pericardium. Other diseases were excluded by laboratory examinations. The isolated strains proved to be *Mycoplasma anatis*.

GENETIC ANALYSIS OF *MYCOPLASMA GALLISEPTICUM* STRAINS

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The polypeptide and DNA restriction patterns of six *Mycoplasma gallisepticum* strains from various parts of the world were compared. All polypeptide patterns but one were identical after staining the separated proteins by Coomassie blue dye. Obvious differences in polypeptide patterns of the strains were demonstrated by fluorography performed after the electrophoresis of radiolabelled mycoplasma proteins. Further and even more pronounced differences were obtained by DNA restriction analysis. Based on these data the six strains were assigned to four groups. Our data and previous findings demonstrate that a precise characterization and classification of mycoplasma strains must be based on their genetic analysis.

EVALUATION OF DIFFERENT MEDIA FOR THE CULTIVATION OF *UREPLASMA*

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A total of 135 frozen specimens positive for *Ureaplasma urealyticum*, were recultured on A 7B agar, GM agar, Shepard U 9C broth and SP-4U broth. The number (%) of recoveries were: Shepard U 9c, 15 (11.1); GM + A 7B, 26 (18.8) and A 7B, 86 (63.7); SP-4U 135 (100.0). A 7B agar can be used for primary plating, and SP-4U broth appears to be a useful enrichment medium.

INTERACTION BETWEEN *LEPTOSPIRA INTERROGANS* SEROVARS *POMONA* AND *CANICOLA* IN HAMSTERS

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To evaluate the immunizing potency of experimental leptospiral vaccines 134 vaccinated and 78 unvaccinated (control) Syrian hamsters were infected with *Leptospira interrogans* serovars *pomona* and *canicola*. Both highly virulent strains adapted to hamsters by serial animal passages were lethal for them, but the susceptible hamsters infected with a few of cultivated leptospires survived and harboured the leptospires in their kidneys sometimes for six months. These *pomona*-infected and survived hamsters were resistant to the subsequent lethal *canicola*-reinfection, and vice versa, the *canicola*-infected ones survived the lethal *pomona*-reinfection. As there was no antigenic relationship between *L. interrogans* serovars *pomona* and *canicola*, the protection against antigenically heterogeneous leptospires was assumed to be due to the common pathobiological properties. This cross-immunity was not demonstrated in hamsters vaccinated previously with killed or avirulent leptospires, but in those previously infected with sublethal doses of virulent heterologous leptospires it was present for three months against *pomona* and five months against *canicola*. The duration of the homologous immunity in survived hamsters lasted at least for year.

ELISA FOR THE DETECTION OF *BRUCELLA OVIS* INFECTION

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ELISA has been developed for the detection of antibodies against *Brucella ovis* using heat-extracted and sonicated antigens. Examining 399 samples from four flocks, ELISA proved to be more sensitive than the complement fixation and the AGID tests. The overall agreement of ELISA using antigens produced in different ways was 99.5%. The background values of negative sera in ELISA using a sonicated antigen were higher than those using a heat-extracted one. This had no effect on the interpretation of the results. Sonication of the same quantity of bacteria yielded more antigen than heat-extraction.

GNOTOBIOTIC PIGLETS AS MODEL ORGANISMS FOR EPIZOOTIOLOGICAL STUDIES

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Gnotobiotic pigs were immunized at 16, 26 and 34 days of age with killed antigens of exotoxin producing *Bordetella bronchiseptica* or *Pasteurella multocida* strains. Pigs of a third group were infected at the same age with 5×10^8 live, avirulent *B. bronchiseptica* organisms by intranasal route. Apart from regular bacteriological examination, the systemic (humoral and cell-mediated) and the local immune response were followed up by appropriate tests. Cell-mediated immune response was determined by lymphocytic blast transformation, and the immunoglobulin content of the blood serum and nasal discharge by agglutination, precipitation and indirect immunofluorescence tests. The results indicated that despite poorer haematological parameters compared to conventional piglets, the gnotobiotic piglets can be advantageously used as model organisms for epizootiological studies.

THE ROLE OF A *BORDETELLA* *BRONCHISEPTICA* CYTOTOXIN IN THE PATHOGENESIS OF TURBINATE ATROPHY IN PIGS

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Bordetella bronchiseptica produces turbinate atrophy in pigs and predisposes the nasal cavity to colonization by toxigenic *Pasteurella multocida*. *B. bronchiseptica* strain B58 was shown to produce in vitro an haemolysin, an adhesin, adenylate cyclase, a mouse lethal factor and a cytotoxin; none of these factors were produced by a phase III variant of strain B58. Another isolate, PV6, produced the haemolysin, adhesin and adenylate cyclase but not the mouse lethal factor or cytotoxin. Strains B58 and PV6 colonized the nasal cavity of gnotobiotic pigs in large numbers during an infection experiment whereas the phase III strain colonized in small numbers. However, only the cytotoxic strain, B58, caused turbinate atrophy. These results suggest that the haemolysin, adhesin and adenylate cyclase were not the cause of turbinate atrophy seen in these pigs.

FIMBRIAE OF *MORAXELLA BOVIS* AND *NEISSERIA OVIS* ISOLATED FROM CATTLE

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Biochemical properties and haemagglutination of 19 *Moraxella bovis* and 48 *Neisseria ovis* (*Moraxella ovis*) strains isolated from the conjunctiva of healthy and of cattle affected with keratoconjunctivitis were tested. Four strains of each were investigated also by electron microscopy for the presence of fimbriae. *N. ovis* causes mainly conjunctivitis in calves, while from cases of keratoconjunctivitis of beef cattle both *M. bovis* and *N. ovis* can be cultured. Based on morphological and biochemical properties, *M. bovis* can clearly be differentiated from *N. ovis*. In contrast to *N. ovis* isolated from sheep, none of our isolates agglutinated red blood cells of cattle, sheep, horse, rabbit, guinea pig, chicken or man, but on the surface of both bacterial species fimbriae were seen. *M. bovis* had relatively few but long fimbriae while *N. ovis* fimbriae were numerous and their length did not exceed the diameter of the bacterial cell.

BACTERIAL INFECTIONS OF THE GENITALIA OF RAMS IN HUNGARY

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Occurrence and role of three Gram-negative bacteria (*Brucella ovis*, *Histophilus ovis* and *Actinobacillus seminis*) infecting the genitalia of rams were studied. Out of 828 semen samples collected from 32 flocks in North Hungary, 181 *B. ovis*, 54 *A. seminis* and 45 *H. ovis* were isolated and examined during the last two years. Most of the semen samples, altogether 705 were derived from mature breeding rams. The number of semen samples examined from one flock varied from 3 to 78. Thirty flocks were found to be infected with *B. ovis*, 21 with *A. seminis* and 17 with *H. ovis*. All *B. ovis* isolates were cultured from rams either with epididymitis or with subclinical genital infections. Within a flock the number of rams shedding *B. ovis* varied from 2.2 to 36.3%, but if semen samples were collected from clinically ill or serologically positive rams, *B. ovis* could be cultured from 73.9 to 100% of the samples. *A. seminis* and *H. ovis* infections occurred sometimes also in mature breeding

rams. However, in young virgin rams *H. ovis* and *A. seminis* are the main cause of infections of the genitalia, while *B. ovis* was cultured from three semen samples only.

SEROGROUPS OF *CAMPYLOBACTER* STRAINS ISOLATED FROM BOVINE AND OVINE FETUSES

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Campylobacter strains isolated from aborted bovine and ovine fetuses of different herds were characterized biochemically and serologically. For the heat stable antigens indirect haemagglutination test (IHA) was used. From *Campylobacter fetus* strains boiled alkaline extracts were prepared and used as antigens. Out of 48 bovine isolates causing abortion, 46 represented *C. fetus* subsp. *venerealis*, of which 44 were assigned to serogroup O1 or A and 2 strains to serogroup O2 or B. The two further isolates belonged to *C. fetus* subsp. *fetus*, serogroup O1 and to *C. jejuni* Penner's serotype 1, respectively. For the serological examination of *C. fetus* strains boiled alkaline antigen extracts can be used in the IHA test in the same way as for Penner's serotyping of *C. jejuni*. Out of the ovine isolates 28 represented *C. fetus* subsp. *fetus*, of which 16 were assigned to serogroup O1 and 12 to serogroup O2. Six *C. fetus* subsp. *fetus* strains grew well at 25, 37 and 43 °C as well. Further 21 isolates were identified as *C. jejuni*; 7 of them represented Penner's serotype 1, while the remaining strains could be assigned to three further serotypes.

RESTRICTION-MODIFICATION LIKE PHENOMENON OBSERVED IN *MYCOBACTERIUM* *SMEGMATIS* STRAINS ARE CONSEQUENCES OF NATURAL POLYLYSOGENY

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Titration phage butyricum (By) on its original host, *Mycobacterium smegmatis* butyricum, and on *M. smegmatis* SN2 (SN2) phenomena referring to restriction-modification could be observed. On the contrary, observations

showing that By phages propagated on SN2 cells have altered morphological structure — instead of the icosahedral shape of the head of phage By, the head of phage By propagated on SN2 cells is elongated — could not be explained by the effect of DNA modification. It has been assumed that the phenomena observed resulted in consequence of natural lysogeny of the *M. smegmatis* strains. This hypothesis was proved by biological methods and electron microscopic observations. Phages with non-contractile tails of both types of head morphology were found to be present in lysates of *M. smegmatis* butyricum and SN2 cells, showing the polylysogenic property of these strains.

PRIMARY STRUCTURE OF *ESCHERICHIA COLI* R₁ LPS DOES NOT DETERMINE THE SEROLOGICAL SPECIFICITY OF THE MOLECULE

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In order to determine the immunodominant region of LPS molecule[?] lipopolysaccharides from mutants of *Escherichia coli* R₁ and R₄ type with very similar or identical primary chemical structure but very different serological activity have been compared. First of all, affinity of terminal galactoses to galactose-specific lectines and its reactivity with galactose oxidase have been determined. Serological reactivity of R LPS dissolved in dimethylsulfoxide, could be eliminated and again reconstituted by change of pH. This phenomenon could be attributed to conformational changes within the molecule.

DETECTION AND DETERMINATION OF RARE 2-AMINOSUGARS AND 2-AMINOOURONIC ACIDS DERIVED FROM BACTERIAL LIPOPOLYSACCHARIDES

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Gas chromatographic method has been elaborated for separation of all eight of the possible 2-aminoaldohexose epimers. By the use of this method, it was possible to determine 2-aminouronic acids when amino compounds were released by methanolysis rather than hydrolysis and the aminouronic

acids yielded were reduced to aminosugars. Effectiveness of the method has been demonstrated on lipopolysaccharides derived from *Shigella sonnei*, *Micrococcus lysodeikticus*, *Vibrio parahaemolyticus* and *Methanobacterium thermoautotrophicum*.

EFFECTS OF THE ENANTIOMERS OF TRICYCLIC PSYCHOPHARMACONS ON *ESCHERICHIA COLI* PLASMID MAINTENANCE AND FUNCTIONS OF HUMAN LEUKOCYTES IN VITRO

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Stereoisomers of tricyclic psychopharmacons e.g. methotrimeprazine and clopenthixol exert antibacterial, F⁺lac plasmid curing and R-plasmid transfer inhibiting effects which are independent from steric symmetry in *Escherichia coli*. Basic properties of molecule structures e.g. configuration and conformation referring to antibacterial and plasmid curing effects differ according to lipohydrophobicity, directions and strength of dipole moments and charge transfer-complex forming ability of the molecules. Members of stereoisomer pairs had no immunosuppression, but the inhibition of certain functions of human leukocytes showed some stereoselectivity.

INVESTIGATION OF ANTIBACTERIAL AND ANTIPLASMID EFFECTS OF TRICYCLIC COMPOUNDS IN VITRO BY QSAR ANALYSIS

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The correlations between the antibacterial effect, plasmid elimination and physicochemical parameters of 9 phenothiazine and anthracene derivatives were studied by QSAR analysis. The dipole moments, the x, y, z vectors of the dipoles, and the lipophilicity (log P) of the compounds were involved in the study. The fit of the data computed by linear multiple regression was controlled via the statistical parameters. The data proved that the antibacterial effect is more dependent on the lipophilicity of the compounds than

the plasmid elimination itself. The former effect depends on the dipole vectors x and z , while the plasmid elimination depends on vectors x and y . In both cases the biological activity increases with the negative sign of the dipole vectors. The correlations between the data suggest that the bonding sites of the compounds in question are nucleophilic.

IMPORTANCE OF *LISTERIA MONOCYTOGENES* IN FOOD HYGIENE

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Since the WHO/ICFMH/IUMS Meeting on "The Present International problems in Food Microbiology" (Budapest, 1983) events occurring in the world made the repeated dealing with the problems of listeria infections justified. The WHO also decided to discuss the questions of prevention and control of listeriosis in December, 1986. Now it is accepted that *Listeria* may be a food-borne pathogen for man. Pathogenetical problems of the microbe, possibilities of its spread, circumstances of sporadic diseases should be studied and epidemiological data should be uniformly collected and evaluated. Further important tasks are to determine optimal conditions of food-production, food-industry, trade and technique of home-cooking for the protection against this infection as well as to promote organization of protection of members of risky groups.

SEVEN YEARS EXPERIENCE OF HOSPITAL SCREENING EXAMINATIONS

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Hygienic conditions in one of the hospitals of Pécs have been examined since 1979. More than 2000 different samples (settle-plates, swabs, surface contact plates, finger-print cultures, sterility control) were collected from its Surgical and Traumatology wards, Burns Unit, E. N. T. Department and from their operating theatres. A similarity was observed between the results of cultivation of settle-plates and swabs when samplings were performed on a suitable way. It is not reasonable to use surface contact plates for sampling

because the results of this method have not given more useful informations than those of the before-mentioned two methods. Presence of enteric bacteria in an operating theatre or in a ward suggest serious hygienic insufficiency. On the basis of the results a new standard is proposed for hygienic classification. Bradosept proved to be an effective hand desinfectant when it was used properly. The examinations offered a possibility to evaluate the hygienic situation objectively and had an educational effect.

BACTERIOLOGICAL EXAMINATION OF THE DISINFECTING EFFECT OF HOSPITAL EQUIPMENT

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The disinfecting effect of a bedpan washing machine (Elektrolux) and dishwasher machine (Miele G-7735, G-7736) was examined. Cultures of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Straptococcus faecalis* ($1.28-5.04 \times 10^8$ cfu/ml) were placed inside the machines. The viable counts were determined after exposure to the heat-disinfection process. The disinfecting effect of the hospital equipments was satisfactory as well as the applied method of testing.

CRYPTOSPORIDIOSIS: OBSERVATIONS ON ANIMAL AND HUMAN HEALTH SIGNIFICANCE

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Cryptosporidiosis was diagnosed by microscopic examination of faecal or intestinal samples of young diarrhoeal farm animals and of symptomless workers of some infested farms. Faecal or ileal smears were stained by modified Castaneda or by Kinyoun method. Intestinal samples of calves, lambs and of goats were also studied by EM. The prevalence of cryptosporidiosis was: humans, 2/78 (2.5%); pigs, 4/289 (1.4%); calves, 131/714 (18.3%); lambs, 10/53 (18.9%); goat kids, 36/104 (34.6%). Presence of cryptosporidia was concomitant with diarrhoea in animals but not in man. However, in another patient without any reported animal contact, suffering from AIDS-related

diarrhoea, cryptosporidia contributed to the clinical signs. Cryptosporidiosis occurred in calves as mixed enteric infections with coronavirus, rotavirus or K99⁺ *Escherichia coli* in 60% of the cases. Rota- and adenovirus complicated the cryptosporidiosis in some goat kids. *Entamoeba coli* accompanied cryptosporidia in the two farm workers. It is concluded that cryptosporidia should be regarded as significant enteric pathogens in young ruminants but not in piglets or in immunocompetent adult persons. The data also indicate their zoonotic potential.

IDENTIFICATION OF VIRIDANS STREPTOCOCCI ISOLATED FROM CLINICAL SPECIMENS

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A total of 2721 strains of viridans streptococci isolated from clinical specimens were identified by using a scheme based on the work of Rotta and Facklam. The distribution of the *Streptococcus* strains was: *S. milleri* 26.1, *S. mitis* 61.3, *S. mutans* 3.6, *S. salivarius* 3.8, *S. sanguis* 3.0 and *S. uberis* 2.2%. The conventional methods and a micromethod for the determination of fermentation spectrum, aesculin hydrolysis and arginine decarboxylation gave practically identical results on 1840 strains. A shortened scheme based on Facklam's was successfully used to identify viridans streptococci.

EVALUATION OF THE WELLCOME REVEAL COLOUR STREP A TEST

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The test demonstrating the presence of *Streptococcus pyogenes* antigens in throat swabs within 5 min has been applied for different groups of patients: (1) suspected cases of scarlet fever to make an early diagnosis; (2) scarlet fever contacts to identify group A streptococcus carriers; (3) children with undiagnosed tonsillitis and pharyngitis; (4) children in paediatric wards with cured group A streptococcus infections. The specimens were taken with cotton swabs and not with the plastic ones made by Wellcome. Out of 150 specimens *S. pyogenes* could be demonstrated in 22 by culture and in 19 by Strep A test. Positive results obtained by the Strep A test were always confirmed by culture. The Strep A test may be recommended for screening in outbreaks in communities and hospitals.

EFFECT OF AMNIOTIC FLUID ON GROUP B *STREPTOCOCCUS*

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Publications of the antibacterial effect of the amniotic fluid date from the 1960-s. In the period 1982–84, we found that some amniotic fluids inhibited bacterial multiplication after the 32nd week of pregnancy. The antibacterial action of amniotic fluids on group B streptococci was determined after 0, 1, 2, 3, 4, 5 and 24 h exposure. Of the amniotic fluids (from the 37th–40th week of pregnancy) 23 were effective and 51 ineffective. The phenomenon was attributed to antibacterial substance(s) in the amniotic fluid. Supplementary toxicity tests on the amniotic fluids were carried out on monkey kidney (Vero) cell line. Neither the effective nor the ineffective amniotic fluids proved toxic.

LEGIONELLOSIS IN A CLOSED AIR CONDITIONED WORK PLACE

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Within a short period of time, 3 severe cases of pneumonia were revealed among employees working in a closed, air conditioned place. On the basis of clinical features legionellosis was suspected. In one patient's pleura exudate bacteria reacting with *Legionella pneumophila* type 1 specific antiserum were detected with direct FA test. His serum and sera of the other two patients showed indirect immunofluorescence in significant titres. Extensive investigations in the community revealed two more serologically positive cases. Rods with typical morphology were detected by type 1 immunofluorescent staining from samples of the water supply system and from the water of the air conditioner. Culture of the samples was unsuccessful probably because of a routine disinfection short before water sampling.

DIAGNOSTIC POSSIBILITIES OF CONTAMINATION OF HUMAN *CHLAMYDIA TRACHOMATIS* AND THE RESULTS OF ANALYSIS

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Urethral discharge of patients in the Department of Urology were cultured then the pathogenic agent was detected in contaminated cells with FITC signed monoclonal antibody. In part of the specimens chlamydia antigens were detected. Positive result was found in 62% by cultivation, while direct detection of chlamydia antigens was positive in $\frac{1}{3}$ of cases.

CHLAMYDIA TRACHOMATIS ASSOCIATED WITH DACRYOCYSTITIS IN THE INFANT

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Between January 1 till May 15, 1987, 22 infants suffering from mono- or bilateral dacryocystitis accompanied by muco-purulent conjunctivitis, were examined. In 15 cases *Chlamydia trachomatis* was cultured from the conjunctival specimens. Bacterial contamination occurred in all cases except one. Neisseriae were absent. Probing of tear-ducts proved unnecessary, as both purulent dacryocystitis and the conjunctivitis vanished on irrigation of the tear sacs and instillations with rifampicin. Screening of the mothers for chlamydia infection is proposed.

ISOLATION OF A LGV STRAIN OF *CHLAMYDIA TRACHOMATIS*

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A strain of *Chlamydia trachomatis* was isolated in chick embryo yolk sacs from a patient of Ethiopian nationality, hospitalized at the dermatovenerological department, with an acute form of lymphogranuloma venereum. This is the first record of the disease and the first isolation of this chlamydial serotype in Czechoslovakia.

PRODUCTION AND MICROBIOLOGICAL EXAMINATION OF POWDERED PEPTONE FROM "BOVICOR" MEAT EXTRACT

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The Animal Trade and Meat Industrial Company of Baranya County placed on the market a deep-freeze meat extract called "Bovikor S" for preparing culture media. A powdered peptone produced from this material was as good as other home-made or foreign peptones.

SUSCEPTIBILITY OF ANAEROBIC BACTERIA TO NINE ANTIMICROBIAL AGENTS

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One hundred and eighty-three clinical isolates of anaerobic bacteria were tested against 9 antimicrobial agents by the reference agar dilution method of the American NCCLS. At their usually achievable serum levels chloramphenicol, carbenicillin, metronidazole proved to be the most effective agents inhibiting all the strains tested except 52% of Gram-positive, non-spore-forming bacilli resistant to metronidazole and 6% of peptococci. Only occasional strains from *Bacillus fragilis* group, *Bacteroides* sp., *Clostridium* sp., some peptococci show resistance to cefoxitin and clindamycin. Ampicillin and penicillin G appear to be very active except for *B. fragilis* (with 58% and 50% of resistant strains at the accepted breakpoints). Tetracycline and erythromycin display the lowest activity and the highest percent of resistant strains among all the species of the studied anaerobic bacteria.

EVALUATION OF MICROBIOLOGICAL FINDINGS IN WOMEN WITH ASYMPTOMATIC BACTERIURIA

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Urine cultivation records for 1036 specimens taken from 19-75 years old women were analysed; of the samples 600 originated from pregnant and 436 from non-pregnant persons. The most important bacteriological patho-

gens were: *Escherichia coli*, pregnant 66.7%, non-pregnant 55.3%; *Proteus* group pregnant 10.4%, non-pregnant 56.7%; *Enterobacter*, pregnant 10.4%, non-pregnant 14.4%. The others, as *Streptococcus faecalis*, *Alcaligenes faecalis*, *Pseudomonas aeruginosa*, *Klebsiella* and *Citrobacter* were present in lower numbers.

FREQUENCY OF *CAMPYLOBACTER PYLORIDIS* ISOLATED FROM PATIENTS WITH GASTROINTESTINAL DISORDERS

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This poster presents a prospective study designed to look at the incidence of *Campylobacter pyloridis* on a series of 158 unselected patients endoscoped for upper gastrointestinal symptoms. *C. pyloridis* was sought as follows: (i) using 1% aqueous basic fuchsin for staining tissue smears; (ii) inoculating the specimen onto blood agar and home made campylobacter selective medium; all plates were incubated in Gas-Pak system without a catalyst at 37 °C and examined after four days; (iii) crushing the biopsy material into 0.5 ml Christensen's 2% urea broth. *C. pyloridis* was isolated from 118 patients (74.6%); by direct examination of smears they were positive in 94% (111/118). An early urease-positive activity in antral biopsy specimens was observed in 90% (107/118) of the infected patients. *C. pyloridis* was isolated from 89.5% (77/86) of the patients with histological evidence of gastritis, 3/10 of the patients with gastric ulcer and 36/38 of those with duodenal ulcer. *C. pyloridis* was detected only in one of 16 patients with normal antral mucosa.

A MONOCLONAL ANTIBODY-BASED LATEX TEST FOR THE DETECTION OF *NEISSERIA* *MENINGITIDIS* GROUP B POLYSACCHARIDE

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A sensitive latex reagent was developed based on monoclonal antibody for the detection of 2-8 linked sialic acid polysaccharide in patient body fluids with *Neisseria meningitidis* group B and *Escherichia coli* K1 infection. The

reagent could detect polysaccharide at 15 $\mu\text{g/ml}$. In a clinical evaluation antigen was detected in the CSF of all 7 *N. meningitidis* group B and 2 *E. coli* K1 infected patients whereas 57 control CSF samples were negative. Cultures from 21 *N. meningitidis* group B and 6 *E. coli* K1 strains gave a positive test and 43 strains of other *N. meningitidis* groups were negative. The reagent was sensitive and specific with potential application in culture identification and antigen detection in infections due to these pathogens. Under modified test conditions, a sialopeptide with the same carbohydrate structure found in brain tissue of neonates could be detected at 14 $\mu\text{g/ml}$ CSF samples from 5 neonates with no evidence of bacterial infection and one adult with neurodegenerative disease gave weak positive reaction with the latex reagent.

DETECTION OF MYCOPLASMA ANTIGENS IN VAGINAL SPECIMENS

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The 4-layer modification of ELISA and the immunobinding technique on nitrocellulose membrane were used to determine *Mycoplasma hominis* directly from vaginal discharge. Cultivation results in each case corresponded to the results of ELISA and nitrocellulose membrane methods. By the use of nitrocellulose membrane the presence of *M. hominis* could be shown in 4 h as contrasted to the long cultivation period.

INVESTIGATION OF NEWLY SYNTHESIZED POTENTIAL ANTIMALARIAL COMPOUNDS IN MICE INFECTED WITH RODENT MALARIA PLASMODIA

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All of 7 primaquine derivatives retained the antimalarial activity, their toxicity decreased considerably and the NADP-derivate completely lost its toxicity. The primaquine NADP-complex seems to be promising for further experiments and eventually for human therapeutic administration.

CHARACTERIZATION OF FREE-LIVING AMOEBAS ISOLATED FROM PATHOLOGICAL SAMPLES AND ENVIRONMENT

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Acanthamoeba castellanii and *Acanthamoeba polyphaga* were isolated from meningoencephalitis and ulcerative keratitis. Many other free-living amoebas were isolated from environmental swimming-pool, river and the soil samples. The isolated amoebas were characterized by morphological examination of the trophozoites and cysts, pathogenicity to mice, antigenic structure and isoenzyme profile compared with PAGE. The morphological, biological and serological markers proved the phylogenetic relations between the free-living amoebas isolated from the pathological samples and the milieu.

Immunology

EFFECT OF HUMAN ADENOVIRUS INFECTION ON THE PRODUCTION OF LYMPHOKINES IN MICE

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The infection of mice with human adenovirus type 6 resulted in immunosuppression. As the virus exerted immunosuppressive effect only in mice injected intraperitoneally, the role of peritoneal cells in the adenovirus induced immunosuppression was suggested, however, it was not demonstrated which of the cellular functions was impaired. In this study the activity of peritoneal cells in the ADCC was tested and it was found that in the adenovirus infected mice the ADCC increased 1, 3 and 7 days after the infection. At the same time a decreased IL-2 production of the spleen cells in the adenovirus infected mice was observed. However, the interferon producing capacity of the spleen cells of the virus infected mice was comparable with that of the control animals when Sendai virus was used as interferon inducer. In agreement with relevant other observations, the data presented suggest that in virus-induced immunosuppression the impaired IL-2 production should be considered.

EFFECT OF PHORBOL-MYRISTATE-ACETATE ON IL-2 PRODUCTION BY HEAT-INACTIVATED *STAPHYLOCOCCUS AUREUS* INDUCED HUMAN LEUKOCYTES

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Heat-inactivated *Staphylococcus aureus* induced IL-2 production similar to that induced by concanavalin A or staphylococcus enterotoxin A. IL-2 preparations induced by different inducers had similar physicochemical and biological characteristics. The staphylococcus-induced IL-2 production was enhanced by the simultaneous addition of phorbol-myristate-acetate (PMA), the effect of which was optimal at 10 ng/ml. In the presence of 10 ng/ml PMA, the amount of staphylococci required for maximal IL-2 production was lower.

The kinetics of staphylococci and staphylococci plus PMA-induced IL-2 production were similar. Stimulated mononuclear cells produced IFN besides IL-2. The titre of accompanying IFN was also enhanced in cultures stimulated with the staphylococci plus PMA combination. The IL-2-producing cells belonged in the plastic-non-adherent fraction of the mononuclear cells. OKT-4 monoclonal antibody and complement treatment inhibited the IL-2 production of this cell fraction.

PRODUCTION OF RECONSTITUTED VIRAL ENVELOPES FOR VACCINATION

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To develop viral vaccines that are free of nucleic acids but still retain the immunogenic properties and potential of viral particles, Newcastle disease virus (NDV) and pseudorabies virus (PRV) models were used. Purified envelope proteins were incorporated into liposomal membranes of phosphatidylserine and -inositol large multilamellar or small, unilamellar vesicles. Inactivated viral particles, purified viral proteins and these liposomes were used for the vaccination of groups of animals, with and without oil adjuvant. Haemagglutination inhibition and ELISA were used for the evaluation of antibody titres. Virosomes provoked equal to or higher titres than immunization with viral particles. High titre immunity prevailed longer in case of virosomes than virus induced immunity. A further advantage of the liposome-mediated vaccination is the total absence of local necrotic lesions at the sites of injections.

SYNERGISTIC PROTECTIVE EFFECT OF ANTIBIOTIC AND IMMUNOGLOBULIN AGAINST *KLEBSIELLA PNEUMONIAE* INFECTION

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In investigating the synergistic protective effect of antibiotic and intravenous immunoglobulin against *Klebsiella pneumoniae* infection, randomized CFLP and NMRI mice of 16–18 g body weight were inoculated intraperitoneally (i.p.) on several levels with different concentrations and dilutions of

cefexitin and immunoglobulin. The mice were challenged with *K. pneumoniae* K15 strain (10^7 bacteria, in 0.5 ml, i.p.). The pathogenicity-promoting effect of the strain was increased with ferric ammonium citrate. The course of the infection process was followed by estimating the live cell count. A significant synergistic protectivity could be demonstrated. Depending on the dilution levels, the synergistic effect was more pronounced if the immunoglobulin was given prior to the antibiotic.

EFFECT OF IRRADIATED ENDOTOXIN (TOLERIN) ON THE CELLULAR IMMUNE RESPONSE ABILITY OF GERM-FREE AND CONVENTIONAL MICE

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Cellular immune response was examined to intracerebral LCM virus infection in germ-free (Gf) and conventional (Cv) young adult mice pretreated with Tolerin. The Gf and Cv C3H mice were treated with 1 mg/kg and 10 mg/kg doses of Tolerin, then the day following treatment they were infected with 100 LD₅₀ dose of LCM virus. One mg/kg Tolerin treatment had no effect either on the course of LCM virus infection or on the lymphoid organs in both Gf and Cv mice. In Gf mice treated with 10 mg/kg Tolerin and infected with LCM virus, the mortality rate was high and they died earlier than the untreated ones. Cv mice infected with LCM virus died in 100%, whereas those pretreated with Tolerin died earlier. Ten mg/kg Tolerin treatment thus facilitated the course of LCM virus infection in the form of meningitis, i.e. it enhanced cellular immune response. Parallel with the deaths following LCM virus infection, significant spleen hypertrophy evolved in Gf and Cv mice treated with 10 mg/kg Tolerin. This dose had no effect on the peripheral blood-count or the thymus of mice. By the end of the treatment, on the 21st day, spleen hypertrophy had ceased to exist.

PHYSIOLOGICAL AND REGULATORY PARAMETERS OF GERM-FREE MINIPIGLETS

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Slower maturation of the immune system is one of consequences of bacterial antigen absence in germ-free (GF) minipigs comparing to CV controls. To take in account other aspects, we studied alpha-fetoprotein (AFP) natural immunomodulator in fetal and early postnatal life, and neuroendocrinologically active compounds with possible immunoregulatory effects, such as beta-endorphine and cortisol. There was a higher blood serum concentration of AFP until the 21st day of postnatal life in GF minipiglets compared to CV controls. Higher AFP content can affect various immune reactions including immunosuppression of T and B functions and inhibition of phagocytosis. Ontogenetic enzyme profiles of Na^+K^+ -ATPase, Mg^{++} -ATPase and gamma-glutamyl transpeptidase activities were estimated on lymphocytes of GF and CV animals, indicating slower cellular maturation. A decreased blood serum level of beta-endorphine in GF minipigs was estimated.

IMMUNOLOGICAL AND BIOCHEMICAL MARKERS OF GERM-FREE MINIPIGS

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The aim was to investigate immune system markers, characterizing germ-free (GF) minipiglets compared to conventional (CV) controls. In GF minipigs there was a (i) delayed maturation of secondary lymphatic organs with persistence of extramedullary hemopoiesis; (ii) lower number of lymphocytes in lymphoid organs; (iii) prevailing surface IgM and IgD on B lymphocytes; (iv) lower expression of MHC antigens of class II on macrophages; (v) lower activity of gamma-glutamyltranspeptidase on B-lymphocytes, participating in membrane amino acid transport. All selected markers indicated slower maturation of GF animals in the absence of bacterial antigens. On the other hand, intrauterine immunization of "immunically virgin" pig fetuses with flagellin from *Salmonella* accelerated maturation of the immune system accord-

ing to selected markers including also formation of specific antibodies. Association of GF minipiglets with *Escherichia coli* O86 and *Streptococcus faecalis* var. *liquefaciens* evoked accelerated maturation of lymphocytes observed as dynamic changes of surface properties (enzyme activity).

HAEMATOLOGICAL AND PLASMA BIOCHEMICAL PARAMETERS OF GNOTOBIOTIC PIGLETS

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Parameters were determined of miniature minnesota piglets reared in gnotobiotic conditions of isolators from birth to 14 days of age. Germ-free, monoassociated (*Streptococcus faecalis*, *Escherichia coli*) and conventional (CV) animals were used. All arterial and mixture venous blood samples were obtained from the carotic artery and jugulary vein using heparinized syringes and immediately analysed for pO_2 (7.30–12.50 kPa; 2.05–4.50 kPa), pCO_2 (2.30–6.10 kPa; 5.25–13.35 kPa) and pH (7.2–7.55; 6.95–7.45). Haematological (RBC, haemoglobin, haematocrit) and biochemical (glucose, creatinine, urea, total bilirubin, ALT, AST) concentrations and activities were compared. Significant differences were found between GF and CV piglets in beta-endorphine levels.

THE INTESTINAL ABSORPTION OF COLOSTRAL LYMPHOID CELLS IN NEWBORN PIGS

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The experiments were carried out in 19 newborn pigs of 3 sows (A, B and C). The lymphoid cells of the colostrum were separated using Ficoll-Paque and labelled with technetium ($Na^{99m}TcO_4$). In the 7th h of life, 5 ml aliquots of cell suspensions were injected directly into the stomach (group A) or into the jejunum (group B) following laparotomy, while the members of group C received the cell suspension through a nasal tube. Tissue sections were prepared in a cryostat from the duodenum and jejunum of piglets exsanguinated in the 8th h after treatment. Autoradiography tests proved that

only the maternal colostrous cells were absorbed from the intestines and carried to the mesenteric lymph nodes via the ductus lactealis, while the colostrous cells of foreign sows were observed only in the epithelium of the mucous membrane. According to EM studies the absorption took place intercellularly. Lymphoid cells collected from the peripheral blood did not absorb at all. The results prove that colostrous lymphoid cells can transfer cellular reactivity into the newborns of animals.

IMMUNOCOMPLEXES INDUCED BY VIRAL ANTIGENS IN MICE

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The kinetics of immunocomplex (IC) formation and its connection with the development of humoral and cellular immune response were studied following simple or multiple administration of some viral vaccines. During the primary immune response of mice immunized with Ajjeszky's disease vaccine the IC-s detected by PEG-6000 precipitation, laser nephelometry and/or spectrophotometry appeared in the sera before the appearance of VN antibodies on the 2nd day after immunization. They reached the maximum level on the 6th day and were eliminated from the serum till the 10th day. In mice immunized with Suvac vaccine IC-s appeared later (5th day) and were eliminated sooner (9th day). IC-s formed during the secondary immune response were eliminated from the serum sooner: on the 5th day if the mice were immunized twice and on the 3rd day if they were immunized three times. The kinetics of IC formation and the comparison of the results of the in vitro cellular tests suggested that IC-s formed after vaccination are bound by the immunocompetent cells exerting a helper in the blastogenesis of lymphocytes but not affecting the development of antibody dependent cytotoxic reactions.

MEASUREMENT OF HUMAN TETANUS ANTITOXIN BY ELISA

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The investigations were carried out with respect to practical application of ELISA for detecting tetanus immunity. In principle, the antigen (tetanus toxoid) was immobilized on microplates and served to capture the antitoxin

in human sera. This complex was detected by a rabbit antihuman immunoglobulin G conjugated by alkaline phosphatase. The developed test detected antibody levels of 0.005 IU/ml. However, on conditions that most sera require a dilution for excluding unspecific reactions, the sensitivity of ELISA amounted to levels of 0.03 to 0.05 IU/ml. Comparison of 90 sera resulted in a good correlation between the ELISA and the toxin neutralization test in mice. Additionally, 66 persons necessitating an exact indication of tetanus vaccination for avoiding possible hypersensitivity reaction were tested by ELISA.

DETECTION OF ANTI-*PSEUDOMONAS* *AERUGINOSA* ANTIBODIES IN HUMANS

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An indirect micro-ELISA is under standardization to detect anti-*Pseudomonas aeruginosa* IgG and IgM human antibodies. The typical ELISA titres of the following human sera were determined: (i) hyperimmune anti-*P. aeruginosa* serum as a standard; (ii) high-titre sera of volunteers immunized with *Pseudomonas* vaccine (Pyovac), and (iii) normal serum absorbed with *P. aeruginosa* as negative control. The cross reactions of these sera against other Gram-negative bacterial antigens were tested as well. The ELISA experiments were repeated in the Serum and Vaccine Institute (Prague) for comparison of Hungarian and Czechoslovak anti-human IgG conjugates. The average level of "natural" anti-*Pseudomonas* IgG antibodies in a human population was investigated by testing more than 300 normal human sera to determine the natural "background" of the *Pseuaomonas* ELISA test system.

Mycology

DETECTION OF CIRCULATING ANTIGENS IN *CANDIDA ALBICANS* INFECTION IN HAEMATOLOGICAL DISORDERS

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The inhibition of IHA and ELISA was successfully used in 46 haematological cases. Both the antibody and the antigen titres were measured in the sera. The results indicate the reliability of the technologies as well as the specificity and sensitivity of the tests.

APPLICATION OF A SIMPLIFIED METHOD AND COMMERCIAL KITS FOR THE IDENTIFICATION OF YEASTS

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Traditional procedures for yeast identification are complex and time consuming. Commercial manual kits (API 20C, Yeast-IDENT) and automated computerized systems have been developed offering convenience and reliable identification. All of these systems have been devised to provide identification for only clinically important yeasts. Their databases include only 19–43 species. In contrast, over 200 species have been described from various foods. For these a simplified identification key has been developed applying carefully selected traditional tests. Seventy-two yeast strains freshly isolated from sweet corn were investigated. Twenty species were distinguished by applying conventional methods (auxanographic sugar assimilation, fermentation of glucose, urease, nitrate reduction, microscopic morphology). The simplified key failed to provide identification only in two cases. With the API 20C strips (20 microtests for sugar assimilation) only six species could be correctly identified. The Yeast-IDENT enzyme kit (chromogenic substrates for different glycosidases, lipases, aminopeptidases) allowed species identification only in four cases. The overall performance of the simplified key, API 20C and Yeast-IDENT was 85, 59 and 48%, respectively.

IDENTIFICATION AND CHARACTERIZATION OF SUPPRESSOR MUTANT OF *patl(ranl)* IN *SCHIZOSACCHAROMYCES POMBE* VAR. *POMBE*

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Temperature-sensitive recessive lethal mutations, *patl(ranl)*, that allow haploid sporulation have recently been described (Iino and Yamamoto; Nurse). Now I report on suppressor mutations that precludes this sporulation by conferring defective meiosis (*mei*⁻) or inability to enter the sexual and meiotic pathways (*ste*⁻). All the mutants grow at the restrictive temperature, although they still contain the *patl(ranl)* defect. Complementation analysis revealed that the *mei*⁻ mutants are allelic to *mei2*. The *ste*⁻ mutants were mapped to a new gene, *stell*, and confer a complete block of conjugation, meiosis and sporulation. Thus, the *stell*⁺ gene is supposed to play a central role in the switch between the mitotic cell cycle and alternative developmental pathways.

MEIOTIC EFFECTS OF *cdc* MUTATIONS OF *SCHIZOSACCHAROMYCES POMBE*

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As several events of meiosis and mitosis are the same, cell division cycle mutations may have some meiotic effects. Fourteen *cdc* mutants defective in DNA synthesis and nuclear division were investigated to see whether they are also temperature sensitive for sporulation. None of these genes was directly required in the conjugation process. Mutants *cdc13*, *18*, *10*, *17* and *21* could complete meiosis at the restrictive temperature. Other ones (*cdc20*, *23*, *24*, *25*) could also complete meiosis, but they had less asci. Mutant *cdc2* had two spored asci at the restrictive temperature. According to nuclear staining these spores were diploids. Mutants *cdc6* and *cdc22* could not complete meiosis at the restrictive temperature. Zygotes of *cdc22* and the most of *cdc6* were found to have been blocked with one nucleus. Since all the strains investigated could complete meiosis at the permissive temperature, they are not sterile mutants. Ability to form viable spores was also tested, but none of the examined *cdc* genes was required for it.

REGULATION OF INOSITOL PRODUCTION IN *NEUROSPORA CRASSA*

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Inositol requiring and wild type (inositol independent) strains of *Neurospora crassa* were used for studying inositol synthesis. The effect of inositol and gamma-hexachlorocyclohexane (gamma-HCH) was investigated on different strains. Gamma-HCH was used as an inositol analogue. Myo-inositol-phosphate-synthase (MIPS) — which converts glucose-6-phosphate into inositol-1-phosphate — was followed. Both inositol and gamma-HCH act on the production of MIPS, but have no inhibitory effect upon enzyme activity.

DETECTION OF FOUR SPECIES WITHIN THE *TRICHODERMA HARZIANUM* AGGREGATE BY PROTOPLAST FUSION

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The ability of 65 *Trichoderma* isolates to grow on 127 carbon sources was tested. Cluster analysis was performed on the basis of the data derived from the utilization of the 42 carbon sources which gave reproducible results. Four groups of *Trichoderma harzianum* could be clearly identified. To prove that these groups are indeed distinct species an isolate of each groups was selected and their auxotrophic protoplasts were fused by the PEG-Ca⁺⁺ method in pairwise combinations. No heterokaryons were obtained between the groups, but vigor heterokaryons appeared within a strain if the fusions were made with the same protoplast suspensions. The above results strongly support the idea that at least four species exist within the *T. harzianum* species aggregate.

AN ASPECT OF MYCOCORROSION

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Fungal deterioration of art productions, paintings, codexes (pergamen or paper type), archeologically or historically valuable textiles (cloth, etc.) occur as a consequence of store conditions supporting fungal growth. This

process has been demonstrated on Corvinas (inadequate storage in Turkey), some paintings from the Museum of Arts (pipeline breakage of central heating) and king's cloak (buried for some years).

MICROBIOLOGICAL INVESTIGATION OF THE "JAPAN CRYSTAL"

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The so called Japan Crystal ("White Alga") came to fashion nowadays as a home made "universal medicine" for a wide range of diseases (up to cancer). It is sometimes recommended as a remedy in contradictory symptoms (hyper- and hypotension, hyper- and anacidity, etc.). Macroscopically it is a white, amorphous hard gel grain cca. 4–6 mm in diameter, grown in 1% sucrose solution supplemented with 1–2 raisins. The fermenting liquor is consumed. Microscopically budding yeast-like cells and pseudohyphae as well as short, rod shaped, nonmotile bacteria are seen in the fermenting liquor and within the grains. Upon cultivation yeast strains (up to date 3 different strains, depending on the private source of the sample) and one bacterium were found. Identifications performed until now gave *Saccharomyces cerevisiae* race *prostoserdovii* and race *uvarum*, and *Gluconobacter oxydans* as the members of the symbiosis. Like an earlier similar product (Japan or Tea Fungus) used some twenty years ago for the same purposes, the yeast ferments the sugar to ethanol which in turn is oxidized to acetic acid by the bacterium.

DOES MEIOTIC RECOMBINATION INVOLVE THE SAME LIGASE AS THAT INVOLVED IN MITOSIS?

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The possible existence in yeast of different nuclear ligases led us to ask whether meiotic and mitotic recombination involve the same ligase as that involved in DNA replication. The *cdc17* mutant of *Schizosaccharomyces pombe* is known to be defective under restrictive conditions in ligase activity and has a low level of ligase activity even at the permissive temperature. We could detect an increased level of mitotic recombination in a haploid strain which has a duplication in the *ade6* locus. No detectable change of the level of meiotic recombination could be found in the case of either intergenic or intragenic recombination.

ISOLATION AND CHARACTERIZATION OF CAFFEINE-RESISTANT MUTANT IN *SCHIZOSACCHAROMYCES POMBE*

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A repair system of *Schizosaccharomyces pombe* which is sensitive to caffeine is known. Accordingly it is possible to look for mutants, which become damaged in recombination among caffeine-resistant mutants. The isolated mutants endure higher (about 20%) caffeine concentration than the wild-type of *S. pombe* does. Considering UV sensitivity of these mutants on complete medium, two of them proved to be much more sensitive than the wild-type. Caffeine reduces the survival of CR21 mutant after UV exposure less than it does in the case of wild-type. The CR21 mutant is extremely sensitive to UV exposure, and to caffeine (after UV exposure) and its recombination pathway might be damaged. With the help of further experiments we shall try to decide whether the difference is caused by lesion of caffeine-sensitive repair or something else, for example REC mutation.

FUSION OF CHEMICALLY INACTIVATED YEAST PROTOPLASTS

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Protoplasts of *Saccharomyces cerevisiae* were inactivated with different concentrations of antifungal compounds for various periods. Of the 24 compounds tested *N*-ethylmaleimide proved to be the most efficient: at a concentration of 50 µg/ml for 30 min caused 100% inactivation at high concentration of protoplasts, without altering their genetic composition or lysing them. The inactivation effect is fully reproducible. Such inactivated protoplasts could function as fusion partners. When they were fused with normal (untreated) protoplasts by polyethylene glycol treatment, they produced viable hybrid cells. The analysis of the fusion product from inactivation experiment showed similar result to the control (uninactivated) fusion products. The chemical inactivation method seems to provide a new way to counterscreen fusion hybrids when the introduction of selective genetic markers (e.g. auxotrophic mutation) is impossible or when it can be deleterious to the existing genetic composition of industrial strains.

CENTRIFUGAL SEPARATION OF *SACCHAROMYCES CEREVISIAE* PROTOPLASTS WITH DIFFERENT PLOIDY LEVELS

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Attempts have been made to find methods suitable for centrifugal separation of *Saccharomyces cerevisiae* protoplasts of different ploidy levels. Model experiments were carried out with well marked *S. cerevisiae* strains (di/tri/tetraploids) constructed from polyauxotrophic haploid mutants. For preparations three different centrifugation media (Ficoll, Percoll, Nycodenz) were used. Good results were obtained with continuous gradients of Ficoll and Nycodenz (5–25%) by employing rate zonal centrifugation. Separations based on the buoyant densities of the protoplasts were examined on different Percoll gradients (45–95%). Samples of protoplasts of various ploidy levels were separated, regenerated and analysed. The procedure of ploidy-dependent separation of protoplasts, in certain cases, gives an opportunity to enrich cells of higher ploidy after fusion or after mating, without introducing genetic markers into the partners.

A NEW METHOD TO INCREASE THE REGENERATION FREQUENCY OF *ACREMONIUM* PROTOPLASTS

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Protoplasts of some industrially important *Acremonium chrysogenum* strains have very poor regeneration rates in the universally used agar media. The application of solidifying macromolecules other than agar may increase the regeneration frequencies of these protoplasts. Cryoprecipitate, agarose and Ca-alginate were tested. Cryoprecipitate is a freeze-dried fraction of blood-plasma which contain mainly proteins. Its clotting may be induced by adding Ca-ions. In the regeneration experiments protoplasts were embedded in 1% agarose or Ca-alginate, or in 5–10% cryoprecipitate supplemented with 0.5% CaCl_2 , and plated onto a bottom layer. Osmotic stabilization was carried out by using non-ionic stabilizer such as sucrose, because the high ionic strength of the most frequently used ionic stabilizers prevented blood coagulation as well as gelation of cryoprecipitate. Using agarose or Ca-alginate, 1.5–2 times

better regeneration rates were obtained than in the case of agar as a solidifying agent. The best results were reached with cryoprecipitate. Two to four times more regenerating colonies developed in this medium than on the control plates.

CHARACTERIZATION OF AN INTERSPECIFIC HYBRID OF TAXONOMICALLY DISTANT ASPERGILLI BY ISOENZYME ANALYSIS

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The interspecific hybrid was obtained by fusing the protoplasts of auxotrophic mutants of *Aspergillus nidulans* and *Aspergillus fumigatus* with polyethylene glycol. Following the genetic analysis the hybrid was characterized by isoenzyme analysis. The liquid minimal medium was inoculated with conidia suspension of the parental strains or mycelia suspension of the hybrid. In order to avoid the effect of the catabolite repression in case of some enzymes, the glucose was substituted for Na-acetate, the ammonium-ion for nitrate ion, "Skim milk powder" or uric acid. After three days cultivation the mycelia were harvested, freeze-thawed in three cycle and grounded in a mortar. The cell-free extract was analysed by gel electrophoresis. Nineteen kinds of enzymes were examined. Our results suggest that the hybrid contains the whole *A. nidulans* genom. In a few cases *A. fumigatus* isoenzymes and new allelic forms appeared. These proved that the nuclear fusion had taken place.

COMPARISON OF EFFICACY OF DIFFERENT ANTIFUNGAL AGENTS IN EXPERIMENTAL CUTANEOUS CANDIDIASIS

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Our infectious model was essentially the technique described originally by Van Cutsem and Thienpont in 1971, with slight modifications. Female albino guinea-pigs were intramuscularly injected with 200 mg/kg of alloxan one day prior to the infection. On the day of infection the depilated skin of the animals was infected with 0.025 ml of a 24 h *Candida albicans* culture.

The animals were treated with 0.5 ml of different doses of clotrimazole, miconazole, econazole, tioconazole or nystatin solutions for seven days twice daily and for a further week once daily. The course of infection and therapy was assessed according to an arbitrary scale. The statistical analyses were performed by Mann-Whitney U-test. In this experimental model nystatin and tioconazole treatment proved to be highly effective ($p < 0.05$). Clotrimazole, miconazole and econazole therapy yielded no statistically significant differences between the treated and the control groups.

Virology

TRYPSIN-SENSITIVE SITES IN NATIVE ADENOVIRAL HEXONS

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Native hexon capsomers of human subgroup C adenoviruses (AV-1, AV-2, AV-5 and AV-6) contain different numbers of trypsin-sensitive sites. AV-2 hexon is cleaved at 3 sites per subunit (polypeptide chain) previously mapped on the amino acid sequence by Jörnvall and Philipson (1980). The hexon of AV-1 contains only the rightmost of these sites and only the leftmost site was cut in the case of the hexon of AV-6. Native AV-5 hexon was resistant to trypsin attack but, it may be rendered trypsin-sensitive by mild destabilization. Hexon of other adenovirus serotypes of human, simian, bovine, and avian origin behave in this respect like AV-5 hexon, being resistant to trypsin treatment in native state, but partially cleaved after destabilization. All hexon fragments produced by trypsin cleavage in the native state are held together in pseudotrimetric forms resembling intact hexon capsomers.

STUDIES WITH MONOCLONAL ANTIBODIES RAISED AGAINST BOVINE ADENOVIRUS TYPE 2

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The cross reactivity pattern of eight different monoclonal antibodies (moabs) raised against bovine adenovirus type 2 (BAV 2) hexon was studied using seven different adenoviruses in ELISA. The results were as follows.

<i>Virus</i>	BAV-2A	BAV-2B	BAV-1	HAV-1	OAV-3	BAV-4
<i>Moab</i>						
A12	+	+	—	—	—	—
BB7	+	+	—	—	+	—
BH5	+	+	—	+	+	+
CA12	+	+	+	—	—	—
II. A9	+	+	—	—	—	—
III. B11	+	+	+	—	+	—
IV. F3	+	+	+	+	+	—
IV. F5	+	+	—	—	—	—

Since HAV-1, BAV-1 and OAV-1 belonging to Mastadenovirus group I do not react in ELISA with all BAV-2 hexon specific moabs the existence of epitope differences can not be excluded in the hexon structure, though the hexons of the strains belonging to the same group are otherwise indistinguishable. On the other hand, BAV-4 and BAV-8 belonging to Mastadenovirus group II seem to have some common epitopes on their hexon since one of the moabs raised against BAV-2 is also bound by these viruses.

MONOCLONAL ANTIBODIES AGAINST ADENOVIRUS TYPE 35 HEXON

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Monoclonal antibodies directed against adenovirus type 35 hexon were prepared and studied for their cross-reactivity (CR) with 11 different human and two animal adenovirus hexon in indirect ELISA, passive HA and gel diffusion assay. The specifically bound monoclonal antibodies were detected by HRPO-labelled antimouse Ig, urease-labelled antimouse IgG, as well as by HRPO- and urease-labelled SpA. Classes and subclasses of the monoclonals determined by different methods were IgM, IgG1, IgG2b, and IgG3. Based on the CR of the monoclonals of 37 hybridoma cell line supernates genus, intersubgenus, subgenus, intertype and type specificities were determined. Six of the hybridoma cell lines showing different reactivity patterns were injected into mice to develop ascitic fluids. These six ascitic fluids and 17 others containing Ad35 hexon specific monoclonal antibodies were further studied. Six CR patterns were determined in indirect ELISA and give in HA experiments. Fifteen of the monoclonals reacted only in ELISA suggesting that certain epitope(s) are not available for the monoclonals on the hexons bound to the red blood cells.

ANTIGENIC CHARACTERIZATION OF INFLUENZA VIRUSES BY MONOCLONAL ANTIBODIES

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Three monoclonal antibodies (1D11F4, 4D9C3, 1D11G4) were raised against the haemagglutinin of the influenza A/Singapore/6/86 (H1N1) prototype strain with the standard hybridoma technique. Two of them (1D11F4,

4D9C3) gave positive reaction in the haemagglutination inhibition test though they reacted with different epitopes. These sera did not give cross-reaction with earlier representatives of the H1N1 subtype of influenza. Haemagglutination inhibition property was not presented by the third monoclonal antibody but in the Western-blot technique it linked to the haemagglutinin of the A/Singapore/6/86 (H1N1) prototype strain. Eleven recently isolated strains belonged to influenza A H1N1 subtype were tested with 1D11F4 and 4D9C3 monoclonal antibodies in haemagglutination inhibition test. The former reacted with five, whilst the latter with nine strains.

DIFFERENTIATION OF NEWCASTLE DISEASE VIRUS STRAINS BY MEANS OF MONOCLONAL ANTIBODIES

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Virion projection proteins were isolated from purified preparations of Newcastle Disease Virus (NDV) strains H and LaSota, and were used to raise monoclonal antibodies (Mab). Several Mabs were produced; two of them were suitable for strain differentiation of epizootiological significance. One of these Mabs recognized only the LaSota strain belonging to the avirulent group and identified as LaSota several isolates obtained from broiler flocks with respiratory symptoms. The other Mab reacted with almost all of our NDV strains isolated from chicken; however, it failed to recognize the strains isolated from PMV-1 infected pigeons.

VIROGENE AND ONCOGENE EXPRESSION IN MALIGNANT LYMPHOPROLIFERATIVE DISEASES

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Lymphatic leukemia and lymphoma cells of different types were investigated for the presence of BaEV and SSV/GaLV p30 antigen as well as of myb, myc, ras and src oncoproteins by indirect cytoplasmic IFA and competitive RIA. Expression of SSV/GaLV p30 was observed in O-ALL and B-lympho-

blastic NHL. Product of the myc gene was detected in all types of lymphatic leukemias and lymphomas, whereas the ras- or src-specific proteins were expressed only in O-ALL, T-ALL and T-lymphoblastic NHL. The degree of myc expression was higher in high grade than in low grade lymphoid malignancies. High molecular weight DNAs prepared from human tumour cells were transfected into 3T3 cells. Results of transfection experiments suggest cooperation between myc and ras as well as between myc and src oncogenes.

SEROLOGICAL AND STRUCTURAL COMPARISON OF HUMAN AND SIMIAN AIDS VIRUSES

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Human immunodeficiency virus (HIV) and related simian isolates (SIV) have similar ultrastructural morphology, genomic organisation and tropism for T4 lymphocytes. We have studied the serological relatedness between HIV and SIVagm by immunoprecipitation of viral antigens with homologous and heterologous antisera. Extracts of ³⁵S-cystein labelled HIV-1/H9 cells and SIVagm/Molt-4 cells were used as sources of viral antigens. Moreover, MT-2 cells producing HIV-2 were also used for comparison. Human antisera to HIV-1 were from a Hungarian AIDS patient and from seropositive haemophiliacs and homosexuals. Antisera specific to SIV were obtained from infected green monkeys, rhesus monkeys and sooty mangabees. All but one human sera used reacted with gp120 of HIV-1 but not with the analogous gp130 of SIV. Sera from the three infected monkey species recognized gp130 of monkey viruses but not that of HIV-1. p24 and precursor p55 of HIV and SIV were recognized by antibodies to HIV and SIV. None of the human anti-HIV-1 and the three anti-SIV sera reacted with any viral proteins of HIV-2. Our results show that the envelope glycoproteins of monkey viruses are distinct from those of HIV, but are related to each other. However, core polypeptides seem to be more conserved among human and monkey virus isolates. The clarification of the structural relationship between these primate viruses help to understand their evolution and pathogenesis.

RABBIT CELL CLONES TRANSFORMED BY THE ONCOGENE OF SIMIAN ADENOVIRUS 16

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The DNA-injection microtechnique was used by Zavizion et al. (unpublished results) for the transformation of embryonic rabbit cells and to obtain the so called "Mathilde" lines immortalized by simian adenovirus 16 (SAV-16). "Mathilde" lines 1, 3, 4, 5 and 6 were included in this study. The aim of the study was to determine permissiveness of the cell lines to the human adenovirus type 12. Serial passages of the virus (AV-12) on the different cell lines revealed that no infectious particles are produced by the cells. Even the hexon antigen was absent from the cell extracts, thus the cells were shown to produce amounts of the major capsomer below the level of detection by immunoprecipitation if any. High multiplicity of infection was used in order to detect possible DNA replication. In contrast to previous results, only 2 of the 5 lines supported the replication of AV-12 DNA. Chromosome analysis revealed the hypotetraploid nature of all 5 cell lines which cannot explain differences in DNA replication.

THERAPEUTIC EFFECT OF (E)-5-(2-BROMOVINYL)-DEOXYURIDINE, CAFFEIC ACID OXIDATION PRODUCT, AND TRISODIUM PHOSPHONOFORMATE ON CUTANEOUS HERPES SIMPLEX VIRUS TYPE 1 INFECTION IN GUINEA PIGS

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The influence of (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU), caffeic acid oxidation product (KOP), and trisodiumphosphonoformate (TPF) on the course of the primary cutaneous herpes simplex virus infection was investigated by means of a guinea pig test model. The antiviral substances were applied in an ointment with 10% urea as penetration mediator. Starting the treatment 15 min after virus inoculation, 3% BVDU were effective against the development of herpetic vesicles and 0.1% BVDU against the appearance of herpetic satellites. Under the same conditions 1 and 3% KOP ointments

inhibited the appearance of satellites. TPF ointment (0.5%) completely inhibited the development of cutaneous herpes lesions. Prophylactic drug administration given 24, 20 and 4 h before virus inoculation was without any protective effect.

MODE OF ACTION OF ANTIBIOTICS AND OTHER COMPOUNDS ON VIRUS SYNTHESIS IN THE TRANSFECTION SYSTEM OF *BACILLUS SUBTILIS*

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Investigating the mechanism and kinetics of the transfection of *Bacillus subtilis* with SP50 phage DNA the antibiotics were administered to the transfection system in different phases of the synthetic process which have been monitored by measuring the growth of the host cells and the rate of the virus production. The antibiotics were selected according to the mode of action for the bacteria inhibiting the synthesis of the cell wall, the proteins and the nucleic acids. The chemicals were DNA-complexing agents and mutagens. (i) The inhibitors of the cell wall synthesis lysed the bacteria while the virus synthesis progressed continuously at a lower level. (ii) Antibiotics acting on the protein synthesis did not disturb the growth of the bacteria but blocked the virus synthesis at the level being on the point of the drug administration. (iii) Antibiotics and chemicals acting on the nucleic acids inhibited the growth of the host cells at different rate and completely stopped the synthesis of the viruses. It is suggested that the *B. subtilis* transfection system is a good model for the study of the antibiotics and other chemicals acting on the synthetic process of viruses.

COMPARATIVE RAPID DIAGNOSTIC STUDY FOR ROTAVIRUS DETECTION AND STUDY OF MOLECULAR EPIDEMIOLOGY

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Rotavirus infection has been investigated in acute gastroenteritis by EM, CIEP, IF, ELISA, latex agglutination and RNA electrophoresis. In the last eight years we have found viruses or virus antigens in an increasing pro-

portion from 20 to 38% of the samples. In the first three months of the year 1987, we found 38% of 247 faecal samples positive for rotavirus infection. The sensitivity of the latex kits was higher than the RNA-electrophoresis. The RNA electrophoresis of the samples was performed in polyacrylamide gel. The majority of rotaviruses belonged to the two main electrophoretic groups. The latex agglutination test (Orion Diagnostica, Finland) is simple, rapid and sensitive: the test does not require any special equipment. RNA electrophoresis in polyacrylamide gel is of great importance as a method for the study of the molecular epidemiology of the rotavirus infections.

STUDY OF THE GENOME STRUCTURE OF VIRULENT PSEUDORABIES (AUJESZKY'S DISEASE) VIRUS STRAINS

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In the last few years we have continued the comparative restriction endonuclease analysis of the genome of pseudorabies (Aujeszky's disease) virus strains isolated from Hungarian outbreaks. The genome structure of a total of 110 virus strains isolated in 60 different localities of counties Fejér, Komárom and Hajdú-Bihar was analyzed by restriction enzymes *Bam*HI and *Kpn*I. In agreement with our earlier results, alterations affecting cleavage sites were found only in a few strains. In most cases differences were observed in the variable fragments. Alterations affecting cleavage sites, highly valuable in epidemiological investigation, were found in *Kpn*I fragment C of a strain isolated in county Komárom ($C \rightarrow C1 + C2$), in *Bam*HI fragment 8-8' of a strain isolated in county Fejér ($8 + 8'$ and $8 + 13$ fusion), and in *Kpn*I fragments E-F of a strain isolated in county Hajdú-Bihar ($E + F$ fusion).

GENETIC AND BIOLOGICAL CHANGES IN THE BARTHA VACCINE STRAIN OF PSEUDORABIES VIRUS

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Since the mid-sixties, numerous attenuated vaccines against pseudorabies (Aujeszky's disease) of pigs have become commercially available. One of these is the Bartha (K/61) strain, which is a small-plaque mutant isolated from

a natural outbreak. This vaccine strain is innocuous to pigs and sheep. Though it kills part of the inoculated mice and rabbits, but without symptoms of pruritus and in a longer time as compared to virulent strains. The genome of strain K/61 is a class 2 DNA molecule of the *Herpesviridae* family: the long unique (U_L) region of the genome is not bracketed by repeat sequences and is not invertible. In 1985 we observed that rabbits used for inocuity testing became ill in larger numbers than usual and showed signs of pruritus. Analysis of the genome structure of inocula used for vaccine production revealed that a virion population with an invertible U_L (i.e. belonging to class 3 DNA structure) propagated to high numbers within the K/61 population. This may have been due to passaging in chicken cells. Virions with a genome of invertible L component produce considerably larger plaques in chicken cells and have increased virulence for day-old chicks. The K/61 strain of altered DNA structure is innocuous to SPF piglets.

INVERSION OF PSEUDORABIES (AUJESZKY'S DISEASE) VIRUS DNA ON PASSAGE IN CELL CULTURE

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The herpesvirus genome consists of two covalently linked components, long (L) and short (S). The S component of pseudorabies virus (PrV) DNA is bracketed by inverted repeats. The S component occurs in two orientations, normal and inverted, relative to the L component, giving rise to two isomeric forms of the genome. Herpesvirus genomes with this structure have been designated class 2 DNA molecules. The structure of human herpes simplex virus (HSV) DNA is even more complex: here both the L and S components are bracketed by inverted repeat sequences and both components invert themselves relative to each other, giving rise to four isomeric forms of the genome. Genomes with this structure have been designated class 3 DNA molecules. In this study, PrV strains were passaged in chicken embryo fibroblast (CEF) cell cultures. PrV variants with an invertible L component (class 3 DNA structure) emerged after 10 to 20 passages in CEF. However, no such change occurred even after as many as 70 passages in pig kidney and rabbit kidney cells.

GENOME SEGMENTS RESPONSIBLE FOR LOSS OF VIRULENCE OF THE BARTHA AND TATAROV PSEUDORABIES (AUJESZKY'S DISEASE) VACCINE STRAINS

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The Bartha and Tatarov vaccine strains of pseudorabies (Aujeszky's disease) virus differ in origin and mode of attenuation: the Hungarian K/61 strain is a small-plaque mutant isolated from a natural outbreak (Bartha, 1961), while the Bulgarian vaccine strains (Tatarov, 1968) was obtained by passage in the presence of a mutagen (IUDR) and is a thymidine kinase-lacking (TK⁻) mutant. By recombination with DNA segments from virulent virus, on the genome of the two vaccine strains we identified loci where alterations resulting in the loss of virulence had occurred. Two such loci were found in the genome of the Bartha strain: replacement of these by virulent DNA segments restored the virulence of the vaccine strain but only if both defects were repaired. On the other hand, in the Tatarov strain only one defect was identified (in the TK gene). Replacement of the DNA segment carrying the defective TK gene with the TK⁺ DNA segment restored the virulence of the Tatarov strain, too. The defects responsible for the loss of virulence of the two vaccine strains differ in both localization and function.

RAPID DIAGNOSIS OF HERPES- AND ADENOVIRAL INFECTIONS BY DIRECT FILTER HYBRIDIZATION

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Aujeszky's disease (AD), infectious bovine rhinotracheitis (IBR) and adenoviral infections were detected by a rapid direct filter hybridization method requiring no purification of DNA from the specimens. Virus specific DNA clones corresponding to fragment of pseudorabies, IBR and bovine adenovirus types 2 and 4 were used as probes. The method was, tested on cultured infected cells, on experimentally infected pigs (AD), calves (IBR,

adeno) and lambs (adeno) and on specimens from an outbreak of AD. The analysis of clinical specimens showed concordance with virus isolation and with hybridization of purified DNA, indicating that the direct filter hybridization method is a good tool of rapid diagnosis. The further advantages of the method are (i) it is independent of cell culture facilities, (ii) an easy and fast sample processing, (iii) simplified hybridization procedure, (iv) the diseases are diagnosed within 15 h.

THE MOLECULAR CLONING OF A BOVINE HERPESVIRUS-1 ISOLATE

M. BENKŐ, B. HARRACH, G. MAGYAR, S. BELÁK and A. BARTHA

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Department of Epizootiology, University of Veterinary Science, Budapest, Hungary*

The DNA of a bovine herpesvirus-1 strain freshly isolated from a respiratory disease (IBR) was digested by the restriction enzyme *Pst*I and cloned randomly into pKH47 plasmid. The *Pst*I cuts the genome into at least 30 fragments. After screening over 200 clones by mini plasmid preparation (boiling) method, 35 recombinant plasmids were selected for further investigations, representing more than 80% of the total genome. The viral DNA fragments are being subcloned into protein expression vectors and tested by immunoenzymatic methods for the expression of fragments of viral structural proteins, and also into an "open reading frame identification and expression" vector to identify the genes of some viral proteins. Several plasmids containing one or more different *Pst*I fragments are being tested as possible diagnostic tools in routine DNA hybridization tests.

CHANGE OF VIRULENCE OF PMV-1 STRAINS ISOLATED FROM PIGEONS

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Hungary*

A total of 12 mesogenic Newcastle Disease Virus (NDV) strains isolated from chickens and 11 virus strains isolated from PMV-1 infected pigeons were passaged in 2-3 weeks old SPF chicks. Our aim was to determine whether the virulence of mesogenic virus strains derived from two different hosts changed upon passage in chicks. While most of the mesogenic strains (mainly

vaccine viruses) of chicken origin could be maintained in chicks only up to the 2nd to 4th passage, all PMV-1 strains isolated from pigeons could be passaged in chicks. Virus strains before passage and those recovered from the last chicken passage (2nd–5th) were compared for virulence by determining their intravenous pathogenicity index (IVPI). The virulence of three out of the six PMV-1 isolated remained unaltered even after five chicken passages (IVPI = 0.0, 0.30 and 0.60). On the other hand, the virulence of the other three strains increased considerably (IVPI = 0.0, 1.30; 0.20, 1.30; 0.0, 1.60).

SEROEPIDEMIOLOGICAL SURVEY OF HEPATITIS A VIRUS INFECTIONS

Ö. POHL

National Institute of Hygiene, Budapest, Hungary

More than 8000 blood samples from cases with acute hepatitis, and 3200 blood samples from healthy subjects were tested by indirect immunofluorescence method for IgM and/or IgG antibody to hepatitis A virus (HAV). An increasing incidence of serologically confirmed hepatitis A cases was observed in adolescents (10–14 years) and in young adults (20–29 years). The frequency of healthy persons with anti-HAV IgG antibodies attained 50% only in the group of young adults, and 90% in persons over 60 years of age. A seroepidemiological investigation of 6 outbreaks of hepatitis A in rural schools showed that spreading of infections occur rarely in school settings in Hungary.

INTRATYPIC DIFFERENTIATION OF COXSACKIE B VIRUS STRAINS BY THEIR ADSORPTIVITY TO Al(OH)₃ GEL

ZS. SZÉNÁSI, E. SZÖLLŐSY, G. PETHEŐ, É. VÁNYAI,
I. KISS and B. CSISZÁR

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The aim of this study was to determine the intratypic differences among Cocksackievirus type B1, B2, etc. strains obtained either from cardiomyopathic or from non-cardiomyopathic patients. For this purpose, the adsorptivity of the virion to Al(OH)₃ gel was tested. The adsorptivity was expressed by the numerical value of the molarity of the phosphate buffer causing 50% elution

of the viruses adsorbed on $\text{Al}(\text{OH})_3$ gel (EC_{50}). ^{32}P -labelled, purified virus suspension was used. The EC_{50} values of the 46 strains examined so far fell between 0.012 and 0.260. The $\text{Al}(\text{OH})_3$ gel adsorption method can be regarded as a new tool for intratypic differentiation among Coxsackie B virus strains. Out of 144 serum samples of patients with different diseases 55 showed high antibody titres ($\geq 1:512$), many of them against more than one type of Coxsackie B. From patients with heart trouble some blood samples were taken months after the beginning of the acute symptoms. A high frequency of IgM type antibodies in these cases is remarkable, it suggests that Coxsackie B viruses might be the aetiological agents.

PREPARATION OF PURIFIED ANTIGEN FOR SEROLOGICAL TEST FROM MONKEY ROTAVIRUS (SA-11) SUSPENSION

A. KÁTAI, É. VÁNYAI, G. PETHEŐ, I. KISS and E. SZÖLLŐSY

Public Health Station, Szeged, Hungary

The aim of the study was to produce an antigen preparation suitable for RIA and ELISA. This has been carried out by applying a method used for enteroviruses. The SA-11 was propagated in GNK line supplied with culture media containing ^{32}P . After the appearance of cytopathic effect the cells were ultrasonicated. The virion suspension was processed by combined chromatography on $\text{Al}(\text{OH})_3$ gel and DEAE cellulose, and its homogeneity was checked by elution test from $\text{Al}(\text{OH})_3$ gel (E marker). A 50% equilibrium was brought about by 0.08 mol phosphate buffer ($\text{EC}_{50} = 0.08$). The $\text{Al}(\text{OH})_3$ elution test using unprocessed virion suspension gave the same EC_{50} value if the evaluation was carried out by cytopathic titration. For ELISA a partially purified and concentrated ($\text{CP} \sim 10^{10}/0.1$ ml) virion suspension was produced and applied for immunization of rabbits. The preparations proved suitable for both virus detection in stool samples and in other experimental arrangement for antibody titration.

APPLICATION OF COMMERCIAL MICROELISA TECHNIQUE FOR THE AETIOLOGICAL DIAGNOSIS OF PATIENTS HOSPITALIZED WITH ACUTE HEPATITIS

I. MIHÁLY, E. IBRÁNYI, N. KESZEI, B. LÁSZLÓ and E. NAGY

László Central Hospital for Infectious Diseases, Budapest, Hungary

In the period from November 1985 to December 1986, blood samples of 1041 patients with acute hepatitis, were tested with different Organon Microelisa Hepanostika Kits and marker changes were followed up in all positive cases. Out of 473 patients tested 45.4% proved to have hepatitis A or B. Nine out of 473 positive cases were detected as having positive markers both for hepatitis A and B simultaneously. Real dual infections could be ascertained in three cases, while in the rest the aetiological role of either hepatitis A or B virus could be proven by the follow up tests. In certain cases unusual combinations of hepatitis B markers were observed. These findings were analyzed in respect of actual clinical features.

ISOLATION AND IDENTIFICATION OF AN AVIAN INFECTIOUS BRONCHITIS VIRUS

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and "Március 15" Agricultural Co-operative, Hernád, Hungary*

A coronavirus was isolated in a 3-week-old broiler flock showing severe respiratory signs. The flock had not been vaccinated against infectious bronchitis (IB). Virus isolation was performed in SPF eggs inoculated with the supernatant of tracheal homogenate from chicks that had shown respiratory signs for approximately 3 days. The virus was demonstrated in cryostat sections of tracheas by the direct IF test. During egg passages virus propagation was detected by IF test in smears of the CAMs. Serotyping was carried out by the neutralization of immunofluorescent foci (NIFF) test. The isolate was reacted with antisera against Massachusetts, Connecticut, H52, H120 and the Dutch variant strains. To detect antibody in the flock the haemagglutination inhibition (HI) method was used as described by Alexander. Although information from a one-sided neutralization experiment is limited, it can be stated that the isolate is closely related to the Massachusetts serotype since the Massachusetts type antisera neutralized the new strain. In the HI test a significant increase of titres against M41 antigen was observed.

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CONTENTS

Phosphorus/Phosphonic Acid-Containing Antimicrobial Antibiotics (A Review) <i>Uri, J. V., Bendas, C. M., Burdash, N.</i>	221
The Kinetics of Cell Division in a Synchronizing Fermentor <i>Novák, B., László, E.</i>	259
Changes of Alternative Respiration During the Division Cycle of <i>Candida tropicalis</i> <i>Novák, B., László, E.</i>	269
Ring-Forming, Oligotrophic <i>Microcycilus</i> Organisms in the Water and Mud of Lake Balaton <i>Langó, Zs.</i>	277
Cell-Mediated Immune Response of Goats to Leptospirosis <i>Siddique, I. H., Mohamed, A. A.</i>	283
Subdivision of Common <i>Salmonella</i> Serotypes: Phage Typing of <i>S. virchow</i> , <i>S. manhattan</i> , <i>S. thompson</i> , <i>S. oranienburg</i> and <i>S. bareilly</i> <i>G. László, V., Sz. Csórián, É.</i>	289
Interferon Production in Myelo- and Lymphoproliferative Diseases. I. Spontaneous Interferon Production in Acute and Chronic Leukaemias <i>Szabó, B., D. Tóth, F., Kiss, J., Vácsi, L., Kiss, A., Rák, K.</i>	295
Alterations in the Chemical Composition and Antigenicity of <i>Escherichia coli</i> O89 Lipopolysaccharide and Lipid A after ⁶⁰ Co Gamma Irradiation <i>Elekes, E., Lüderitz, O., Galanos, Ch., Bertók, L.</i>	301
Virulence Associated Plasmid and R-Plasmid Curing in <i>Yersinia enterocolitica</i> by some Tricyclic Compounds <i>Molnár, J., Nakamura, M. J.</i>	307
Epidemiological Patterns and Incidence of Bio-, Sero- and Phage Types of <i>Vibrio cholerae</i> in Hyderabad, India, During 1971-1984 <i>Rathna, K., Khairunnisa, Rajyalakshmi, K., Naidu, A. S.</i>	313
Priming of <i>Staphylococcus aureus</i> -Induced Interferon Production in Human Buffy Coat Leukocytes by Human Interferon-Alpha Pretreatment <i>Rosztóczy, I.</i>	323

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PHOSPHORUS/PHOSPHONIC ACID-CONTAINING ANTIMICROBIAL ANTIBIOTICS

(A REVIEW)

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(Received December 22, 1987)

Introduction and classification of antibiotics	221
Historical background and general features of phosphorus-containing antibiotics	223
The non-phosphonic acid phosphorus antibiotics with antibacterial and/or antifungal activity	224
Antibiotics 11837 RP, 8036 RP and 19402 RP	224
Diumycin	225
Macarbomycin	227
Moenomycin	228
Phosalacine	229
Phosphazomycin A	231
Phosphinothricin and phosphinothricyl-L-alanyl-L-alanine	231
Prasinomycin	232
Proticin	233
Teichomycin A ₁	234
Related compounds	236
The phosphonic acid-containing antibacterial antibiotics	236
Phosphanilic acid	237
Fosfomycin	241
Alafosfalin	246
Fosmidomycin	249
Monophosphams and phosphonic cephalosporins	251
Other than antibacterial phosphorus/phosphonic acid-containing compounds	252
References	253

Introduction and classification of antibiotics

Within the vast array of available scientific literature, several thousand antibiotics and antibiotic-like agents have been reported. Among these, several hundred have been shown to be useful in clinical situations. A variety of classification systems have been developed over the years in order to facilitate a better understanding of these agents and their interrelationships. These various classification schemes differ depending upon their purpose and usage. One good practical approach to classification is to group the agents according to the type of microbe against which they display antibiotic activity. Thus, in such a system, it is customary to speak of antibacterial, antifungal, antiviral, antihel-

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minthic and antitumour antibiotics. The antibacterial agents can be conveniently classified further on the basis of their spectrum of activity. That is, whether they can inhibit the growth of bacteria which are Gram-positive or Gram-negative, aerobic or anaerobic, or any combinations of a variety of different characteristics. In addition, in such schemes, specific compounds (antimycobacterial, antileprosy, antipseudomonal, etc.) have been developed. The terms, antibiotic and chemotherapeutic, will be used throughout this paper in an intentionally broad sense and will apply interchangeably.

Another way of classifying antibiotics keeps in mind the modes of action of the various anti-infective drugs and separates them into those which are bacteriostatic, bactericidal, bacteriolytic or any combinations thereof. Similar classifications which consider the mechanisms of action of groups of antibiotics will classify them as those which are cell wall-active or cell membrane-active compounds and those which inhibit DNA or RNA functions, protein synthesis or selectively important metabolic processes. A more scientific method for grouping antibiotics takes advantage of their biogenetic origins. The biogenetic system of antibiotic classification differentiates compounds on the basis of the origin of their chemical building units. Hence, they may be derived from amino acids, sugars, fatty acids and their metabolic products such as acetic or propionic acids, etc. The shortcomings of this type of classification are that, for many antibiotics, these biogenetic aspects are not yet known or are incomplete and must be based, even if only temporarily, on hypotheses rather than established facts. Whichever of the above methods for antibiotic classification are considered, we will necessarily find many overlaps and exceptions.

Presently, the most scientifically sound and universally acceptable system for grouping the anti-infective agents is based upon their chemical composition and structural features. The most often encountered groups within this scheme and some of their principal representative members are listed below as follows.

1. *Beta-lactam antibiotics* with the subgroups of penicillins, cephalosporins, cephamycins, oxacephamycins, monobactams, penems and carbapenems. This is the most crowded group comprising more than one hundred medically useful compounds, derivatives and combinations. The list is growing and newer preparations are being developed in the research laboratories of the pharmaceutical industry, universities and other institutions [1-4].

2. *Aminoglycosides* such as streptomycin, gentamicin and tobramycin.

3. *Macrolides* such as erythromycin and roxithromycin.

3. *Tetracyclines* such as tetracycline, oxytetracycline and minocycline.

5. *Ansamacrolides* such as rifampin, rifabutin and rifapentine.

6. *Lincosamines* such as lincomycin and clindamycin.

7. *Glycopeptides* such as vancomycin and teicoplanin.

8. *Polypeptides* such as the polymyxins and bacitracin.

9. *Polyene macrolides* such as nystatin, flavofungin and amphotericin B.

10. *Sulfonamides and sulfones* such as sulfadiazine, sulfadoxine, sulfizoxazole and dapsona.
11. *Antifungal azoles* such as miconazole, ketoconazole, itraconazole.
12. *Fluoroquinolones* such as norfloxacin and ciprofloxacin.
13. *Diaminopyrimidines* such as pyrimethamine and trimethoprim.
14. *Nitrosoureas* such as streptozocin, carmustine and lomustine.
15. *Nitrofurans* such as nitrofurazone and nitrofurantoin.
16. *Aminoquinolines* such as chloroquine and primaquine.
17. *Nitroimidazoles* such as metronidazole and benzimidazole.
18. *Steroidal antibiotics* such as fusidic acid, cephalosporin P₁ and helvolic acid.
19. *Anthracyclines* such as doxorubicin (Adriamycin), daunorubicin and rubidazone.
20. *Phosphorus-containing antibiotics* such as fosfomycin, fosmidomycin, alafosfalin, diumycin and prasinomycin.

It is this very last group of compounds in the above list which are the subjects of this review. Although these drug products and/or substances enjoy an increasing interest, they seem not to have captured a well-deserved appreciation. We intend to cover not only those phosphonic acid-containing compounds which are currently used in the therapy of infections or are under clinical development, but also the phosphorus-containing antibiotic agents (phosphoglycolipids, phosphines) which are scientifically, chemically or otherwise interesting.

Generally, abstracts will not be included in our references and, for the most part, we will be drawing on original articles with an occasional reference to summary publications. In addition to the chemical compositions or structures of the individual preparations, we will cover their *in vitro* and *in vivo* spectra of biological activities including human data if such are available. The pharmacokinetics, modes of action and mechanisms of development of resistance in the laboratory or during therapeutic use will also be included and discussed where applicable.

Historical background and general features of phosphorus-containing antibiotics

The phosphorus-containing antibiotics, especially the phosphonic acid-containing compounds, represent an interesting group of antimicrobial agents which is steadily increasing in number. Some of these compounds are produced by total chemical synthesis but as many, or even more, represent products of microbial fermentation (especially of *Streptomyces* sp.). Phosphanilic acid was the first member of the phosphonic acid-containing group to be synthesized. Like the other compounds in the group, it is of low molecular weight and possesses therapeutic potential.

The earlier phosphorus-containing antibiotics, the glycolipids and phosphines, were isolated not only as single entities but also as mixtures of similar compounds with unusual chemical and biological properties. Their antimicrobial activity predominantly covers the Gram-positive bacteria, however, some newer members of the group exhibit activity against both Gram-positive and Gram-negative microorganisms. All members of the group contain carbon, hydrogen, oxygen, nitrogen and phosphorus and most have molecular weights of about 1800 or greater. Only a few have been prepared in crystalline form, especially phosphines which have lower molecular weights.

Only the empirical formula and not the complete chemical structure has been elucidated for most of these compounds. Many have a complicated phosphoglycolipid composition and tend to form aggregates in aqueous solvents. The glycolipid- and phosphonic acid-type antibiotics are known to inhibit bacterial cell wall synthesis. They are dose-dependently bactericidal and are occasionally bacteriolytic for actively growing bacterial cells. Some of these compounds have additional antifungal activities.

For the most part, these compounds possess low acute toxicity in animals and are well-tolerated. In experimental chemotherapeutic studies, when these agents are given parenterally to mice, they are not only curative for Gram-positive bacterial infections but they also possess remarkable prophylactic activities. In addition to their antimicrobial activity, the phosphoglycolipid class of antibiotics were shown to significantly promote the growth of swine, calves and poultry when administered orally as feed additives.

Detailed descriptions of many of the earlier phosphorus-containing antibiotics can be found in excellent summary papers and comprehensive books from which we have drawn abundantly [5-9]. We have updated much of the information with recent additional relevant data and discuss some of the newer compounds which have been reported in the literature. Table I lists the non-phosphonic acid-containing phosphorus antibiotics. Given in this table are the names of antibiotics, the year and place of discovery and the names of the producing organisms.

The non-phosphonic acid-containing phosphorus antibiotics with antibacterial and/or antifungal activity

Antibiotics 11837 RP, 8036 RP and 19402 RP

These compounds are among the earliest phosphorus-containing antibiotics discovered in the Rhône-Poulenc Laboratories and announced in an abstract (11837 RP) and in French patents 1963, 1964 and 1967, respectively [5, 10-14]. Since they were later shown to be identical with, or very similar to, other subsequently published and named phosphorus antibiotics, they will be discussed or recollected later in this connection.

Table I

Non-phosphonic acid-containing phosphorus antibiotics

Antibiotic	Year and place of discovery	Producing strain
Antibiotic 11837 RP	1963, Rhône-Poulenc	NR*
Antibiotic 8036 RP	1964, Rhône-Poulenc	NR
Antibiotic 19402 RP	1967, Rhône-Poulenc	NR
Diumycin	1969, Squibb	<i>Streptomyces umbrinus</i>
Macarbomycin	1970, NIH Japan	<i>Streptomyces phaeochromogenes</i>
Moenomycin	1965, Hoechst	<i>Streptomyces bambergiensis</i>
Phosalacine	1984, Kitasato Univ.	<i>Kitasatosporia phosalacinea</i>
Phosphazomycin A	1985, Inst. Phys. Chem. Res., Japan	<i>Streptomyces</i> sp.
Phosphinothricin	1972, Univ. Tübingen	<i>Streptomyces viridochromogenes</i>
Prasinomycin	1967, Squibb	<i>Streptomyces prasinus</i>
Proticin	1972, Hoechst	<i>Bacillus licheniformis</i>
Teichomycin A ₁	1978, Lepetit	<i>Actinoplanes teichomyceticus</i>

* NR = Not reported

Diumycin

Diumycin has been shown to be a mixture of closely related fractions (diumycins A, B, A' and B') with specific distinguishing chemical and biological characteristics extracted from the mycelial mass of *Streptomyces umbrinus* ATCC 15972 [10-12]. The molecular weights for the diumycin fractions range from 1600 to 1700 and the empirical formulae for their sodium salts are in the range of C₆₈₋₇₂ H₉₃₋₁₀₇ O₃₈₋₄₀ Na₃ P. Diumycins tend to form aggregates with molecular weight of about 32 000 in aqueous solution. The main hydrolysis products of these compounds are glucosamines and a mixture of optically active, non-isoprenoid C₂₅ lipids such as diumycinol, isodiumycinol and diumycene [13].

The in vitro antibiotic activity of diumycin covers Gram-positive bacteria and *Mycobacterium bovis* BCG. The compound exhibits cross-resistance with another phosphorus-containing antibiotic, prasinomycin. Diumycin has relatively little or no activity against Gram-negative bacteria, *Trichophyton mentagrophytes*, *Candida albicans* and *Trichomonas vaginalis*. It also has good antibiotic activity against both penicillin-sensitive and penicillin-resistant clinical isolates of *Neisseria gonorrhoeae*. Table II summarizes the in vitro antimicrobial spectrum of diumycin, giving the minimum inhibitory concentrations (MIC) in µg/ml [11, 12].

In vivo, diumycin was found to be highly effective in mouse infection-protection experiments where a single subcutaneous injection proved protective. The PD₅₀ values (the dose which protected 50% of lethally infected mice from death) were shown to be 0.22 mg/kg for *Streptococcus pyogenes*, 1.0 mg/kg

Table II
In vitro antimicrobial spectrum of diumycin

Test Organism	MIC ($\mu\text{g/ml}$)
<i>Staphylococcus aureus</i> 209P	0.06
<i>Streptococcus pyogenes</i> C203	0.001
<i>Streptococcus pneumoniae</i> ATCC 6303	0.30
<i>Bacillus subtilis</i> ATCC 6633	0.14
<i>Bacillus cereus</i> ATCC 10876	0.05
<i>Micrococcus luteus</i> ATCC 9341	> 25.0
<i>Mycobacterium bovis</i> BCG 5516	3.10
<i>Escherichia coli</i> ATCC 10536	> 25.0
<i>Escherichia coli</i> SC 8294	50.0
<i>Klebsiella pneumoniae</i> ATCC 9997	> 25.0
<i>Salmonella paratyphi-B</i> SC 3850	> 25.0
<i>Proteus vulgaris</i> SC 8504	37.5
<i>Pasteurella multocida</i> SD 6739	0.59
<i>Pseudomonas aeruginosa</i> SD 3840	> 25.0
<i>Pseudomonas aeruginosa</i> SD 8329	31.2
<i>Trichophyton mentagrophytes</i> SC 2637	>100.0
<i>Candida albicans</i> CHS 35H	> 25.0
<i>Trichomonas vaginalis</i> SC 8560	>100.0

for *Staphylococcus* sp. and 5.06 mg/kg for *Streptococcus pneumoniae*. A most remarkable feature of diumycin is its long-lasting effect. It was shown that it protected mice even when it was administered subcutaneously 14 days prior to challenge. Its name, diumycin, reflects this property (*diu* from Latin for long-lasting and *mycin* reflecting its streptomycete origin). Oral administration of diumycin has not been shown to be practical since more than a 2000 times greater dose is needed for limited protection. This is probably due to inadequate absorption from the gastrointestinal tract.

Diumycin has been demonstrated to be a very non-toxic antibiotic in mice. With the exception of transient liver damage, no other significant gross or microscopic pathologic changes were observed in macropsied mice 8 days after dosing. The acute LD₅₀ values (the dose that kills 50% of mice) were found to be 590 mg/kg after intravenous administration and 455 mg/kg after intraperitoneal injection.

Diumycin is strongly bound to serum proteins, a fact which is most likely responsible for the prolonged serum levels and represents a circulating drug reservoir. The serum half-life ($T_{1/2}$) was found to be about 4 days in dogs and 7 days in monkeys, however, the drug was detectable at 20 days or more after intramuscular injection. The aggregate formation previously mentioned may also contribute to its long duration of efficacy. Diumycin is excreted in the urine, but only a small percentage of the original dose can be detected there.

The mode of action of diumycin can be either bacteriostatic or bactericidal depending upon the concentration of the drug, the genus of Gram-positive

coccus tested, the rate and growth-phase of the bacterial population (actively dividing cells of *Staphylococcus aureus* are killed quickly), the incubation temperature and the presence of serum in the test medium.

The mechanism of action of diumycin is the inhibition of bacterial cell wall synthesis and is similar to that of other closely related phosphorus-containing glycolipid antibiotics such as moenomycin or prasinomycin. In the presence of diumycin, the "Park's nucleotide" (uridine diphosphate-*N*-acetylmuramylpentapeptide) accumulates significantly in the medium suggesting that its incorporation during cell wall synthesis in *S. aureus* is blocked by a direct inhibition of the transglycosylase function.

Macarbomycin

Macarbomycin was originally produced by a strain of *Streptomyces chromogenes* isolated from a soil sample (Geneva, Switzerland) during a routine screening for anti-staphylococcal agents [15]. The biologically active material was isolated from the mycelial mass. Purified macarbomycin was shown to have a molecular weight around 1712–1991 and its name was derived on the basis of its macromolecular nature. The elemental analysis of macarbomycin demonstrated the following range of composition: C_{68–79} H_{123–144} O_{41–48} and phosphorus as free acid. Acid hydrolysis of macarbomycin yields glucosamine, glucose and three lipid compounds.

Macarbomycin is stable in aqueous solutions (50–100 µg/ml) between pH 4 and 10 for one hour at 60 °C. It is unstable below pH 3 and in alkaline solution stronger than 0.1 N NaOH.

In vitro, macarbomycin is highly active against strains of *S. aureus*, including those resistant to other antibiotics, with MIC values ranging between 0.05 and 0.40 µg/ml. It is also effective against *Bacillus anthracis* and *Bacillus cereus* with MIC values for both about 0.025 µg/ml and against *Bacillus mycoides* with an MIC of 0.05 µg/ml. Macarbomycin is less active against *Micrococcus flavus* and *Mycobacterium phlei*, exhibiting MIC values for both of 12.5 µg/ml, as well as, *Mycobacterium* 607 with an MIC of 25 µg/ml. The drug has no real activity against Gram-negative bacilli, yeasts or filamentous fungi.

The intravenous LD₅₀ of macarbomycin in mice was demonstrated to be 750 mg/kg compared to 1500 mg/kg for antibiotic 11837 RP. In mice infected with *S. aureus* Smith, macarbomycin injected intraperitoneally at a dose of 5 and 1.25 mg/kg demonstrated 100 and 50% protection, respectively. Subcutaneous dose of 50 or 25 mg/kg resulted in 50 and 25% survival and an oral dose of 200 mg/kg protected 25% of the infected mice.

Macarbomycin has been reported to be an effective growth promoter in animal nutrition, especially when administered to poultry at 30 g/ton of feed and swine at 2 g/ton of feed.

In studies using staphylococci, the mode of action of macarbomycin was shown to be inhibition of cell wall synthesis, especially at the level of the polymerization of *N*-acetylglucosamine and muramic acid pentapeptide [16]. Macarbomycin has been demonstrated to be more effective against episome-harboured *Escherichia coli* strains than it is against the original episome-free strains [17]. This observation might possibly be due to a cell surface effect of some sort.

Macarbomycin is different from moenomycin and prasinomycin, but its identity with one of the diumycin fractions is unclear at present.

Moenomycin

Moenomycin A, C, D, E, F, G, H are a mixture of very similar chemical compounds which form moenomycin (A is the predominant fraction of the mixture). They are produced by various strains of *Streptomyces* sp. such as *S. bambergensis* ATCC 13879, *S. ghanaensis* ATCC 14672, *S. ederensis* ATCC 15304 and *S. geysiriensis* ATCC 15303, during aerobic fermentation. The drug is extracted from the mycelium, purified and chemically characterized [18–22]. The best empirical formula for moenomycin A is: $C_{70}H_{124}N_6O_{40}P$ (free acid). Antibiotic 19402 RP possesses a chemical formula very similar to the one above for moenomycin.

The moenomycins are colourless, amorphous, weak acids which form high molecular weight associates as phosphorus-containing glycolipids. Moenomycin is water soluble with strong acid functions, heat-stable and behaves like a strong surfactant. Acid hydrolysis of moenomycin yields D-glucosamine, D-glucose, phosphonic acid diester, optically inactive lipid residue (moenocinole) and the ultraviolet chromophore, 2-amino-cyclopentanedione-1,3, and its *N*-acetyl derivative, the chemical structures of which are shown in Fig. 1.



Fig. 1. Structures of two acid hydrolytic products of moenomycin: "Chromophore" and its *N*-acetyl derivative

Moenomycin is reported to possess favorable biological properties [18, 20, 23, 24]. Its antimicrobial spectrum mainly covers the Gram-positive bacteria, however, there is some weak activity against certain Gram-negative organisms. Observed MIC values for moenomycin in $\mu\text{g/ml}$ for some representative organisms are *S. aureus*, 0.05; *S. pyogenes*, 0.05; *S. pneumoniae*, 0.30–1.25;

B. cereus, 0.07; *E. coli*, 8.0–250.0; *Pasteurella multocida*, 0.20–3.0; *Proteus vulgaris*, 24.0; *Pseudomonas aeruginosa*, 94.0. The activity of moenomycin is increased at acidic pH. No cross-resistance between moenomycin and penicillin, tetracycline, streptomycin or macrolides has been observed.

In vivo, moenomycin, injected intravenously or intramuscularly at 5 mg/kg, exhibited protection for mice infected with various streptococcal strains. No effect was observed when moenomycin was administered orally suggesting a poor absorption from the gastrointestinal tract, or "first-pass" effect.

The mouse LD₅₀ for moenomycin was found to be 738 mg/kg by the intravenous route. A single 450 mg/kg intravenous dose of moenomycin was well-tolerated by dogs, but a dose of 600 mg/kg resulted in death.

Moenomycin has the ability to stimulate and promote the growth of animals and has been used as a feed additive [18, 25]. In this respect, it was found superior to Zn-bacitracin and chlortetracycline in the nutrition of broiler, swine and calves at doses of 0.5–1.0, 1.0–5.0, and 5.0–12.0 g/ton of feed, respectively.

In studies using *S. aureus* and *B. cereus*, the mode of action of moenomycin was shown to be the inhibition of cell wall synthesis at the same stage of the biosynthetic pathway inhibited by vancomycin and ristocetin [24, 26]. In cell-free systems, moenomycin, like antibiotic 11837 RP and prasinomycin, inhibits a glycan transglycosylation reaction [26].

Phosalacine

Phosalacine is a phosphorus-containing tripeptide which does not truly belong to the family of the glycolipid-type phosphorus antibiotics. The phosphorus-containing glycolipid antibiotics are large molecules without established chemical structures, however, phosalacine has a defined chemical structure which incorporates the compound, phosphinothricin, which will be discussed later.

Phosalacine was first isolated as an amorphous powder from the culture filtrates of *Kitasatospora phosalacinea* KA-338 [27]. Its chemical structure was elucidated [28] and shown below to be phosphinothricylalanylleucine (C₁₄H₂₈N₃O₆P, mw 365), hence the name, "phosalacine" (Fig. 2).

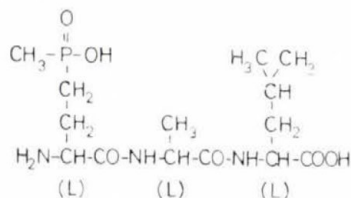


Fig. 2. Phosalacine

Phosalacine exhibits antimicrobial activity against Gram-positive and Gram-negative bacteria and some fungi, but only in chemically defined minimal medium. Table III demonstrates its antimicrobial spectrum (from [27]). The antimicrobial activity of phosalacine is abolished by the presence of L-glutamine in the medium and there is no antimicrobial activity observed in complex organic media. In addition, phosalacine is a potent herbicidal antibiotic. In a concentration of 10 µg/ml it has been demonstrated to kill alfalfa (*Medicago sativa* L.) in four days. Phosalacine is well-tolerated by mice. A single oral dose of 500 mg/kg exhibited no toxic effects.

Table III
Antimicrobial spectrum of phosalacine

Test Organism	MIC (µg/ml)
<i>Staphylococcus aureus</i> FDA 209	0.4
<i>Staphylococcus aureus</i> KB 199	0.2
<i>Bacillus subtilis</i> PCI 219	0.4
<i>Bacillus cereus</i> IFO 3001	0.4
<i>Micrococcus luteus</i> ATCC 9341	6.3
<i>Mycobacterium smegmatis</i> ATCC 607	>100.0
<i>Escherichia coli</i> NIHJ	12.5
<i>Klebsiella pneumoniae</i> ATCC 10031	25.0
<i>Erwinia aroideae</i> KB 148	< 0.05
<i>Pseudomonas aeruginosa</i> KB 139	>100.0
<i>Proteus vulgaris</i> IFO 3167	12.5
<i>Candida albicans</i> KF 1	>100.0
<i>Saccharomyces cerevisiae</i> ATCC 9763	>100.0
<i>Aspergillus niger</i> KF 105	>100.0
<i>Mucor racemosus</i> IFO 4581	6.3
<i>Piricularia oryzae</i> KF 180	0.1
<i>Fusarium oxysporum</i> KF 166	>100.0
<i>Penicillium herquei</i> IFO 7904	>100.0

At present, the mode of action of phosalacine is unclear. The liberated phosphinothricin is most probably the active component. It has been shown to inhibit competitively the enzyme, glutamine synthetase of *B. subtilis* and spinach leaves, much more potently. On the other hand, phosalacine was shown to completely inhibit the growth of *B. subtilis* at a concentration of 0.1 µg/ml, whereas phosphinothricin did not inhibit its growth completely even at a concentration of 50 µg/ml. The mechanism of its herbicidal activity is not known at present.

Other phosphinic acid derivatives, MP-103, MP-104 and MP-105, as well as SF-1293 (bialaphos), are the biosynthetic products of a blocked mutant of *Streptomyces hygroscopicus* [29]. Bialaphos (phosphinothricylalanylalanine) is a herbicide and has the chemical structure shown in Fig. 3 [30].

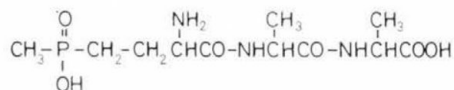


Fig. 3. Bialaphos (phosphinothricylalanylalanine)

Phosphazomycin A

Phosphazomycin is the result of a planned screening program for inhibitors of fungal cell wall biosynthesis [31]. It is extracted from the mycelial mass of a *Streptomyces* sp. as a complex of at least four components active against phytopathogenic fungi. Phosphazomycin A is the main component. It is a colorless, crystalline material with a tentative molecular formula of $C_{37-38}H_{56-60}O_{12-13}NP$. The compound is slightly soluble in water but better soluble in methanol, ethanol and alkaline water.

Phosphazomycin has no antibacterial activity but is a very active growth inhibitor for a variety of fungi and yeasts. Its antifungal spectrum and activity is shown in Table IV (from ref. 31). Inhibitory concentrations were determined by using the conventional agar dilution method [31].

Table IV
Antifungal spectrum and activity of phosphazomycin

Test Organism	MIC (μ g/ml)
<i>Aspergillus oryzae</i>	0.9
<i>Penicillium chrysogenum</i>	0.22
<i>Trichophyton mentagrophytes</i>	0.45
<i>Cochliobolus miyabeanus</i>	0.11
<i>Pyricularia oryzae</i>	0.03
<i>Rhizoctonia solani</i>	7.5
<i>Colletotrichum lagenarium</i>	0.015
<i>Botrytis cinerea</i>	0.008
<i>Glomerella cingulata</i>	0.22
<i>Alternaria mali</i>	0.015
<i>Fusarium oxysporum</i>	30.0
<i>Saccharomyces cerevisiae</i>	15.0
<i>Candida albicans</i>	30.0

The exact mode of action of this compound is not yet known. It has been found to inhibit the beta-1,3-glucan synthetase from *Saccharomyces cerevisiae*. It can produce morphological alteration and its growth-inhibiting effect is accompanied by swelling of the mycelia. In laboratory tests, the antibiotic demonstrated preventive value against cucumber gray mold disease and cucumber anthracnose at a concentration of 25 ppm. The acute toxic amount (LD_{50}) of phosphazomycin in mice was shown to be 19 mg/kg by oral administration.

Phosphinothricin and phosphinothricyl-L-alanyl-L-alanine

Phosphinothricin and phosphinothricyl-L-alanyl-L-alanine are produced by *Streptomyces viridochromogenes* together with other closely structurally related antibiotics [32]. Among them, the tripeptide phosphinothricyl-alanyl-

-alanine, is the main component. The later isolated bialaphos, produced by *Streptomyces hygroscopicus*, is identical to this tripeptide [30] and was isolated from the fermentation broth using precipitation and adsorption to activated charcoal techniques as an amphoteric white powder. Phosphinothricin was obtained in pure form from the hydrolysis of the tripeptide. It is a new amino acid compound containing phosphorus. Its chemical structure was confirmed by total chemical synthesis as 2-amino-4-methyl-phosphino-butyric acid as shown along with its tripeptide (Fig. 4).

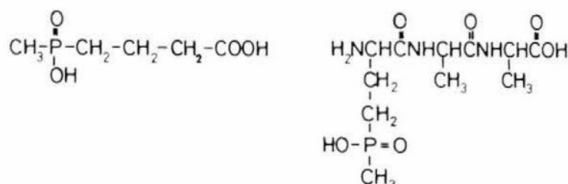


Fig. 4. Phosphinothricin and its tripeptide

The tripeptide is highly active in vitro against Gram-positive and Gram-negative bacteria such as *E. coli* K12, *B. subtilis*, *Pseudomonas saccharophila*, *Streptomyces viridochromogenes* and the fungus, *Botrytis cinerea*. Phosphinothricin itself was found to be less active and only against *B. subtilis* and *P. saccharophila*.

As in the case of phosalacine, the antibiotic activity of this compound is inhibited by the presence of glutamine in the medium. Phosphinothricin is a potent inhibitor of glutamine synthetase, another similarity to the above-mentioned phosalacine. The LD₅₀ of the tripeptide in mice is 50 mg/kg when administered intravenously and 500 mg/kg when given orally.

The mode of action of phosphinothricin and phosphinothricyl-alanyl-alanine is similar to other phosphorus-containing antibiotics, such as phosphonomycin, moenomycin, prasinomycin, diumycin, macarbomycin or phosalacine, in that it is thought to inhibit cell wall synthesis. The tripeptide easily penetrates the microbial cell and inside the cell, phosphinothricin is released, inhibiting the glutamine synthetase with lethal results.

Prasinomycin

Prasinomycins are a complex of at least five antimicrobially active components (A, B, C, D, E) produced by *Streptomyces prasinus* [33] isolated from the mycelial mass after acidification of the culture broth to pH 2. Prasinomycins are of high molecular weight (about 2100–2300) with the following empirical formula C_{62–74} H_{96–130} N_{5–6} O_{32–40} P. Acid hydrolysis has produced D-glucosamine, 6-deoxy-D-glucosamine, glycine, chromophore and a mixture

of optically-inactive C_{25} lipids such as moenocinol [5, 34]. Prasinomycin is a diester of phosphonic acid-containing glycolipid.

The biological study of prasinomycin has demonstrated some interesting activities [35]. In vitro it inhibits primarily the growth of Gram-positive bacteria and *Mycobacterium bovis* BCG (the mixture has the same order of activity as the individual components). The observed MIC values in $\mu\text{g/ml}$ for some pathogens: *S. aureus*, 0.15; *S. pyogenes*, 0.002; *S. pneumoniae*, 6.3; *M. bovis*, BCG, 3.1; *Mycobacterium fortuitum*, 9.4; *Branhamella catarrhalis*, 6.3; *Proteus vulgaris*, 9.4; *Bordetella bronchiseptica*, 12.5; *E. coli*, 37.5; *P. aeruginosa*, 50.0. It can be seen that prasinomycin is more active against Gram-positive than against Gram-negative bacteria and no activity has been demonstrated against yeasts or filamentous fungi.

The in vivo efficacy of prasinomycin is significant. LD_{50} values for subcutaneous administration in mice infected with *S. pyogenes* was 1.0 mg/kg and for *S. pneumoniae* infected mice 6.5 mg/kg. Remarkable is its prolonged protective effect. It has been shown to protect mice against generalized *S. pyogenes* infection for at least eight weeks after a single subcutaneous dose of 200 mg/kg. This same prophylactic activity was observed in mice infected with *S. pneumoniae*. The compound is not active orally and has been demonstrated to be very non-toxic to mice.

The mode of action of prasinomycin is similar to other phosphorus-containing, Gram-positive active antibiotics [36]. It is effective only against actively multiplying cells and is bacteriostatic at low concentrations but bactericidal at higher concentrations. The major mechanism of action of prasinomycin appears to be inhibition of cell wall biosynthesis. Addition of as little as 0.2 $\mu\text{g/ml}$ to growing cultures of *S. aureus* demonstrated an abrupt cessation of incorporation of cell wall amino acids into the mucopeptide and marked accumulation of uridine nucleotide cell wall precursor, both events signs of inhibition of glycan transglycosylation reactions. Prasinomycin appears to be closely related to moenomycin and antibiotic 11837 RP, other members of the family of phosphorus-containing glycolipid antibiotics.

Proticin

Proticin is produced by fermentation of a strain of *Bacillus licheniformis* var. *mesentericus* and isolated in an amorphous form [37, 38]. It contains phosphorus and a conjugated triene and is highly unsaturated with a number of double bonds within its lactam ring. Proticin's molecular formula was determined to be $C_{31}H_{44}O_7PNa$.

Proticin possesses a broad spectrum of antibacterial activity especially against pathogenic Gram-negative organisms. The MIC values in $\mu\text{g/ml}$ for some representative microorganisms are: *P. mirabilis*, 0.40; *E. coli*, 12.0;

Salmonella typhi-murium, 3.0; *Shigella flexneri*, 3.0; *Streptococcus pyogenes*, 0.40; *S. aureus*, 50.0; *Mycobacterium tuberculosis* H₃₇Rv, 5.0. The strong activity of the compound exhibited against *Proteus* spp. is reflected in the name given to the compound, "proticin". Proticin displays a low level of toxicity to mice with LD₅₀ values >150 mg/kg intravenously and 1000 mg/kg when it is injected subcutaneously.

Teichomycin A₁

Teichomycin A₁ together with teichomycin A₂ are produced by *Actinoplanes teichomyceticus*. They are extracted from the fermentation broth and/or the mycelial cake as whitish amorphous powders. Teichomycin A₁ is a new member of the phosphoglycolipid-type antibiotics [14, 39] and will be described here, whereas, teichomycin A₂ belongs to the glycopeptide group of antibiotics (see p. 235 of this review).

Teichomycin A₁ is the first example of a phosphoglycolipid antibiotic produced by a member of the genus *Actinoplanes*.

Teichomycin A₁ is soluble in water, dimethylformamide and dimethylsulfoxide. It is a large molecule with a calculated molecular weight of 3255. Its empirical molecular formula has been determined to be C₁₄₂H₂₄₅N₁₂O₆₉P. Acid hydrolysis of teichomycin A₁ displays a somewhat different pattern than that of the older phosphoglycolipid antibiotics. It contains the common glucosamine residues and an other as yet unidentified sugar moiety. Teichomycin A₁ like other phosphoglycolipid antibiotics, is characterized by the ability to form aggregates in solution. The size of these aggregates is dependent upon the pH and polarity of the solvent.

The basic comparative chemical data for the phosphoglycolipid antibiotics are shown in Table V (taken from reference [14]). The comparative in vitro

Table V
Chemical data of phosphoglycolipid antibiotics

Antibiotic	P%	Glucose	Glucose-amine	2NH ₂ -2,6-di-deoxy glucose	Other neutral carbo-hydrate	Glycine
Moenomycins	1.7—2	+	+	+	+	NR*
Diumycins	1.89—1.91	+	+	—	NR	—
Prasinomycins	2.43—2.28	NR	+	+	NR	+
Macarboxomycins	2.12	+	+	—	NR	+
11837 RP	2.25	NR	NR	NR	NR	NR
8036 RP	2.2	NR	NR	NR	NR	NR
19402 RP	2.4	NR	NR	NR	NR	NR
Teichomycin A ₁	0.96	—	+	—	+	—

* NR = not reported

antibacterial activities of these phosphorus-containing glycolipid antibiotics are presented in Table VI. This table is taken from reference [5] and updated by us. The figures indicated for MIC values should not be taken as absolute since the determinations have been performed in various laboratories using different experimental conditions such as inoculum size, media, pH, incubation, and probably not using the same strains of test organisms.

Table VI
In vitro antibacterial activities of phosphoglycolipid antibiotics

Test Organism	MIC Values in $\mu\text{g/ml}$							
	Diamycin	Macarbo- mycin	Moeno- mycin	Prasino- mycin	8036 RP	11837 RP	19402 RP	Teicho- mycin A_1
<i>Bacillus subtilis</i>	0.14	—	0.1	—	30	3	20	—
<i>Bacillus cereus</i>	—	0.025	0.07	—	0.01	—	0.01	—
<i>Staphylococcus aureus</i>	0.06	0.05	0.05	0.09	0.5	0.2	0.1	0.05
<i>Streptococcus pyogenes</i>	< 0.002	—	0.05	0.24	0.01	0.005	0.1	0.5
<i>Streptococcus pneumoniae</i>	0.3	—	0.3	—	0.08	0.3	—	0.5
<i>Escherichia coli</i>	> 25	50—500	8—250	—	35	—	—	10.0
<i>Neisseria gonorrhoeae</i>	—	—	—	—	0.6	1.5	2.5	—
<i>Pasteurella multocida</i>	—	—	0.2—3	—	2.5	1.5	0.8	—
<i>Proteus vulgaris</i>	—	—	24	25	165	165	125	—
<i>Pseudomonas aeruginosa</i>	—	—	94	≥ 50	45	40	40	—
<i>Salmonella paratyphi-B</i>	> 55	—	—	≥ 50	70	30	60	—

Teichomycin A_1 is very active in vitro against Gram-positive pathogenic bacteria and possesses acceptable activity against certain Gram-negative bacilli. This is in contrast to the activity of teichomycin A_2 which has been shown to be exclusively directed against Gram-positive microorganisms. Representative MIC values for teichomycin A_1 in $\mu\text{g/ml}$: *S. aureus* strain Tour, 0.05; *S. pyogenes*, 0.50; *S. pneumoniae*, 0.50; *Proteus vulgaris*, 20.0; *E. coli*, 10.0. Teichomycin A_1 displays no activity against *P. aeruginosa*, *Candida albicans*, *Trichophyton mentagrophytes*, *M. tuberculosis* H₃₇Rv or *Mycoplasma gallisepticum*.

Teichomycin A_1 injected subcutaneously has been shown to cure mice experimentally infected with lethal inocula of *S. pneumoniae* or *S. pyogenes* with ED₅₀ values of 11.8 mg/kg and 2.5 mg/kg, respectively. It has proven to be non-toxic as well since a dose of 500 mg/kg injected intravenously in mice did not cause the deaths of the animals.

Teichomycin A_2 , now called teicoplanin, has been used clinically against Gram-positive infections and seems to offer some advantages over vancomycin.

Related compounds

Ensanomycin, produced by *Streptomyces cinnamomensis* and *Streptomyces melanogenes*, and prenomycin, produced by *Streptomyces ambofaciens*, are purified materials similar to diumycin, moenomycin or prasinomycin [40] previously discussed.

Another phosphorus-containing antimetabolite produced by a *Streptomyces* sp. is a tripeptide, L-(N⁵-phosphono)methionine-5-sulfoximinyl-L-alanyl-L-alanine (Fig. 5). It is similar to the phosphinothricin-tripeptide discussed

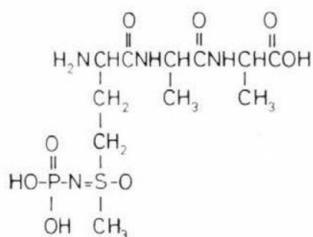


Fig. 5. L-(N⁵-phosphono)methionine-5-sulfoximinyl-L-alanine

earlier and exhibits some antibacterial activity against *B. subtilis*, *Micrococcus glutamicus*, *Streptomyces cellulosae*, *E. coli*, *Serratia* sp., *Enterobacter aerogenes* and *Pseudomonas ovalis* but is inactive against filamentous fungi or yeasts. As with phosphinothricin, its activity is abolished by the presence of L-glutamine in the medium. Its mechanism of action is thought to be similar to that of phosphinothricin and its tripeptide [41].

For the most part, the previously discussed phosphoglycolipid and phosphine-type antibiotics have not been used in human therapy but some of them have been used as growth-promoting agents in animal health and husbandry. They do, however, contribute to a better understanding of antibiotic biosynthesis and modes of action especially regarding the inhibitors of microbial cell wall synthesis nonetheless.

The phosphonic acid-containing antibacterial antibiotics

The phosphonic acid-containing antibiotics are relatively small molecules some of which have been completely synthesized on the basis of rational reasoning while others are microbial products resulting from intelligent screenings of microorganisms. Many of these naturally occurring compounds were later synthesized as well. As a group, they are very non-toxic agents and some have found their way in the treatment of a variety of human bacterial infections.

Others in this group are in clinical trials and are being explored for eventual use in human therapy. These agents are known to inhibit bacterial cell wall synthesis.

Three major phosphonic acid-containing antibiotics have been developed in addition to the earlier phosphanilic acid. They are fosfomycin, fosmidomycin and alafosfalin which will be discussed in detail in this review. Other, less-studied compounds will be only briefly mentioned here. Table VII lists this group of antibiotics and Fig. 6 presents their chemical structures.

Table VII
Phosphonic acid-containing antibacterial antibiotics

Antibiotic	First Described	Origin
Phosphanilic acid	Synthesis : 1941; Activity : 1942	Synthetic (analogy)
Fosfomycin	1969	<i>Streptomyces</i> spp., Now synthesis.
Alafosfalin	1978	Synthetic (design)
Fosmidomycin	1980	<i>Streptomyces lavendulae</i>
Monophosphams	1984	Semisynthetic
Phosphonic cephalosporins	Recent; unpublished	Semisynthetic

Certain aspects of some of these antibiotics have been discussed previously in short summary reviews [6, 7, 40–43]. These references have been used for the preparation of this present, more comprehensive review which will contain updated information concerning these compounds.

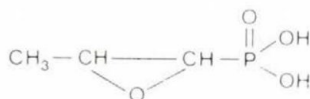
Phosphanilic acid

Phosphanilic (p-aminobenzenephosphonic) acid was synthesized based upon the analogy of sulfanilic acid with the expectation of having similar antibacterial activities as sulfanilamide and the sulfonamides. It is a small molecule with a molecular weight of 173.11. Its chemical structure is shown in Fig. 6.

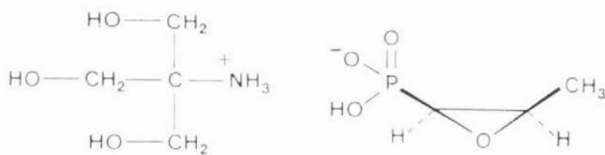
The first organophosphorus compounds were synthesized on the supposition that they might have antibacterial activity and be less toxic than the arsanilic acids. Some of these phosphines did demonstrate activity against streptococcal infections in mice with a low toxicity [44]. The goal to obtain a benzene phosphorus derivative containing a free primary amino group in the para position (similar to the sulfonamides) was fulfilled in 1941 and the name, phosphanilic acid, was suggested by analogy with sulfanilic and arsanilic acids [45]. Later, a new synthesis was described which made phosphanilic acid and related organophosphorus compounds readily available [46].



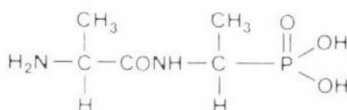
Phosphanilic Acid



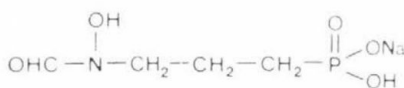
Fosfomycin



Tromethamine salt of Fosfomycin



Alafosfalin



Fosmidomycin

Fig. 6. Phosphonic acid-containing antibiotics

The first detailed chemotherapeutic study of phosphanilic acid was done in 1942 [47]. It compared phosphanilic acid with sulfonamides and sulfones for tuberculostatic activity *in vitro* and for therapeutic efficacy in experimental animals (guinea pigs and rabbits) infected with human or bovine strains. Phosphanilic acid was found to exhibit good *in vitro* inhibition but it demonstrated an irregular effect in the retardation of the progress of the disease *in vivo*. The reason for this variable efficacy was shown to be the poor absorption of

sodium phosphanilate from the gastrointestinal tract and its rapid elimination from the blood after intravenous or subcutaneous injection. Excretion of the drug begins soon after injection. In the first three hours, blood concentrations fall to one-tenth after intravenous injection and one-fourth after subcutaneous administration of the starting level and 80–90% of the drug is eliminated in the urine as free acid within twenty-four hours. The urine becomes acidic and contains large amounts of crystals, made up presumably of the insoluble acid.

The toxicity of phosphanilic acid has been found to be low. Rats were demonstrated to tolerate one gram per kilogram intravenously while no adverse effects were observed in cats upon intravenous infusion of 2 g/kg as a 5% solution of the sodium salt. The above-mentioned pharmacological effects of phosphanilic acid have been confirmed in guinea pigs, mice and dogs [48]. No blood levels of phosphanilic acid could be found following oral administration of large doses of the drug but it could be detected in the feces and urine. This observation has led to the recommendation that it could be used in intestinal infections. Following oral administration, no sign of irritation of the stomach or vomiting was seen in dogs. Mice were shown to tolerate 15 mg three times a day for ten days with a slight loss of weight and appetite and some disturbance of fur as the only adverse reactions displayed. These all returned to normal when the drug was discontinued. Histopathologic examination of the organs of dosed animals showed only slight damage of liver cells and all other organs appeared to be unaffected.

In a short note [49] repeating an earlier report [50], it was demonstrated that phosphanilic acid was only slightly less active against *E. coli* than was sulfanilamide. This activity was shown to be antagonized by p-aminobenzoic acid reflecting another similarity of phosphanilic acid to sulfanilamide.

In another study comparing the inhibitory activity of phosphanilic acid with eight of its derivatives against *E. coli*, *S. aureus*, *S. typhi-murium*, *Shigella boydii* and *Corynebacterium xerosis* in liquid and on solid media [51], it was shown that, with the exception of *C. xerosis*, all of the test organisms were inhibited by the compounds under study with phosphanilic acid being the most effective. Unfortunately, all of the derivative compounds, with the exception of one, were substituted at the p-amino group, the integrity of which is known to be essential for the antibacterial activity of the sulfonamides. Phosphanilic acid has also been shown to inhibit the in vitro growth of *M. tuberculosis* at a concentration of 1 : 5000 [48].

A very important observation concerning the activity of phosphanilic acid showed that this compound was highly active in vitro against *Pseudomonas fluorescens* and *Salmonella typhi* at a concentration of $0.8 \text{ mol} \times 10^{-4}$. Other Gram-negative bacteria were also shown to be susceptible, but to a lesser degree, whereas, Gram-positive bacteria were only slightly, or not at all, susceptible to the action of phosphanilic acid. In a study testing phosphanilic acid and four-

teen of its chemical derivatives, those substituted at the para-amino position proved to be less active against all of the 15 bacterial strains tested. A direct comparison of phosphanilic acid and sulfathiazole demonstrated that their antibacterial activities were qualitatively similar but possessed marked quantitative differences. Phosphanilic acid is much more active against the Gram-negative bacilli such as *E. coli*, *Klebsiella pneumoniae*, *P. vulgaris*, *S. dysenteriae*, *S. typhi* and *P. fluorescens*, than is sulfathiazole. Whereas phosphanilic acid only slightly inhibited the growth of *S. aureus*, sulfathiazole was extremely active against this organism. The same was true in the case of *Vibrio cholerae* and *Bordetella bronchiseptica*. None of the phosphonic acid-containing compounds displayed any inhibitory effects against *Trichophyton mentagrophytes* or *Cryptococcus neoformans*. Phosphanilic acid was also found to be effective in the prophylaxis of *S. typhi* and *P. fluorescens* infections in mice.

Phosphorus-containing compounds, especially phosphanilic acid, have proven to be remarkably non-toxic. The LD₅₀ of phosphanilic acid in mice was found to be 9 g/kg injected subcutaneously in contrast to an LD₅₀ of 1.7 g/kg for sulfathiazole [52].

The potent antipseudomonal activity of phosphanilic acid both in vitro and in vivo in rodents initiated a study of its bio-availability in laboratory animals (rat and dog) and humans with basically similar results [53]. The only significant difference was that in human and rat urine, in addition to the original compound, about 12–15% of the dose was excreted as the antibacterially inactive *N*-acetylphosphanilic acid derivative. The derivative was absent in the urine of the dogs suggesting an interspecies difference.

Phosphanilic acid (potassium phosphanilate) is poorly absorbed orally in all species tested. In humans after 400 and 800 mg oral doses, the serum levels of the drug are low, however, the concentrations in the urine were found to be several times higher than the MIC value obtained for *Pseudomonas* strains. Because of this fact, it may be useful in the treatment of Gram-negative (especially pseudomonal) urinary tract infections, but not for systemic infections.

In dogs, no clinicopathological findings were observed after daily oral administration of 150–450 mg/kg phosphanilic acid for 30 days. In humans, during Phase I studies, no adverse side effects due to drug administration were observed. Vital signs, clinical chemistries, urine analyses and haematology studies remained within normal ranges.

The in vitro antibacterial activity of phosphanilic acid in combination with neomycin or streptomycin has been studied. The combination with neomycin was significantly better than the combination with streptomycin against *Salmonella* sp., *Proteus* sp. and coliform bacilli. Both combinations acted synergistically against *Shigella* sp. [54].

A valuable and impressive study compared the activity of phosphanilic acid alone and in combination with trimethoprim or sulfamethoxazole in vitro

(454 bacterial strains employed) and in the *in vivo* mouse model (6 bacterial strains tested) [55]. Phosphanilic acid was selectively very active against *P. aeruginosa* strains (but not against other *Pseudomonas* spp.) with MIC values of 0.5–2.0 µg/ml (range 0.13–32.0 µg/ml). There was a slight improvement in these values in the case of the trimethoprim combination with MIC values of 0.5–1.0 µg/ml (range 0.25–8.0 µg/ml). In these studies, the activity of phosphanilic acid was influenced by the inoculum size. There was some synergistic activity between phosphanilic acid and trimethoprim against the other bacterial strains employed in the study. The concentration of phosphanilic acid in the blood of mice after intramuscular injection was high enough for therapeutic use but its half-life was short. The blood level after oral administration was low as well. Phosphanilic acid given orally protected mice infected with *P. aeruginosa* with a PD₅₀ found to be 42 mg/kg. The PD₅₀ for the combination with trimethoprim was 43 mg/kg. This observation is unipressive as is the intramuscular therapeutic efficacy.

To overcome the inoculum and medium effects, and the development of resistance in *P. aeruginosa*, phosphanilic acid has been combined with the antibacterial acridines (especially proflavine) with some promising preliminary results [56].

The high level of activity exhibited against *P. aeruginosa* and the high safety profile of phosphanilic acid deserves the preparation of many more derivative of this compound with the promise of expected high therapeutic usefulness.

Fosfomycin

Fosfomycin (formerly phosphonomycin) is an interesting and useful broad spectrum antibiotic originally isolated from several strains of streptomycetes (*S. fradiae*, *S. wedmorensis* or *S. viridochromogenes*). It is the first phosphonic acid-containing antibiotic to be used parenterally. Originally a natural product, it is now produced by total chemical synthesis. It is a small, water-soluble, acidic molecule with the formula C₃H₇O₄P (L-cis-1,2-epoxy-propylphosphonic acid) and a molecular weight of 138.06. The disodium, calcium or lysine salts are water-soluble, but the trimethamine salt has better oral absorption. The structural formula of both are shown in Fig. 6. An unusual feature of the fosfomycin molecule is an epoxide ring, a structure rarely found among antibiotics.

Fosfomycin was found to be active against a variety of Gram-positive and Gram-negative bacteria, not only *in vitro*, but it was also found to be protective to mice infected with those microorganisms. It possesses low toxicity, is bactericidal and inhibits cell wall synthesis. It is widely used in human ther-

apy mostly in Europe and Japan. All of these previously mentioned features, properties and usages will be discussed below.

Fosfomycin was originally discovered and described as the product of the three streptomyces strains mentioned above [57, 58]. It was later isolated from a culture of the bacterial organism, *Pseudomonas syringae* [59]. Its chemical structure was soon established and its total synthesis completed [60].

Most of the in vitro studies on fosfomycin have been done with a preparation produced by fermentation [61], but since its structure is identical to that of the chemically synthesized compound, it should not make any difference in the interpretation of any studies whether done with synthetic or natural fosfomycin.

The in vitro activity of fosfomycin is not great but does cover many Gram-negative and Gram-positive bacteria. The observed MIC values can vary greatly according to the composition of liquid or solid media, inoculum size, pH, etc. and may be 8 µg/ml or 250 µg/ml for the same test strain. The bacterial strains were found in various studies [58, 62–68] to be susceptible to fosfomycin, the MIC values (µg/ml) being: *S. aureus*, 6–12; *E. coli*, 6.25; *P. aeruginosa*, 6–12; *Neisseria gonorrhoeae*, 12; the MIC values were lower than 64 µg/ml for *P. mirabilis*, *S. marcescens*, *Streptococcus* sp., *H. influenzae*, *P. multocida*, *S. paratyphi-B*, *S. enteritidis* and *S. flexneri*.

For some *Salmonella* strains, the observed MIC values can be as low as 1.6 µg/ml. Within the literature there exist much controversial data and great variability. Susceptibility testing with great care and judgement should be done in each case (susceptibility test disks containing 50 µg of fosfomycin are available for such testing). There are *E. coli* and *P. mirabilis* strains which are inhibited by a concentration of 10 µg/ml of fosfomycin. For many strains, this figure is much higher and could be as high as 100 µg/ml, a concentration that can be easily obtained in the urine following oral administration of 2 g/day of fosfomycin. In addition, the usual bacteriostatic concentration is the same as the bactericidal concentration which explains the efficacy of this compound and its low toxicity in urinary tract infections. The development of resistance to fosfomycin is another matter which will be discussed later.

For the in vivo, experimental mouse infection-protection studies, products prepared by chemical synthesis have been used. For subcutaneous and intravenous use, the disodium salt has been employed while for oral administration the calcium salt has been used [65, 69]. Fosfomycin was found to be relatively non-toxic to mice. It is rapidly absorbed from the gastrointestinal tract of the mouse and reaches peak serum concentrations within 0.5–1.0 hour. It is also rapidly excreted in the urine with 50% of the original dose recovered during the first eight hours after oral administration. Fosfomycin has been demonstrated to protect mice infected with the following microorganisms: *S. aureus* (including penicillinase-producing strains), *S. pneumoniae*, *S. pyogenes*, *E. coli*,

P. mirabilis, *P. aeruginosa*, *S. paratyphi-B* and *P. multocida*. In the case of the last two organisms listed, protection was achieved even when fosfomycin was administered four hours after infection.

Representative values for fosfomycin ED_{50} (in mg/mouse) obtained against different bacterial strains are as follows: against *E. coli* administered subcutaneously 2.3–4.5 and administered intravenously 2.1–5.7; against *P. mirabilis* administered subcutaneously 0.3 and administered intravenously 0.2; and against *P. aeruginosa* administered subcutaneously 1.2–6.0 and administered orally 0.6–6.0. Although there are variations in these values, it is important to note, that the effective protective values (ED_{50}) are much better than could be expected from the observed in vitro inhibitory concentrations (MIC).

The pharmacokinetic parameters of fosfomycin have been studied in rabbits' sera and interstitial fluids analyzed after intravenous doses of 20, 30 and 60 mg/kg [70]. Binding to serum proteins was not observed and it was eliminated in the urine unchanged as expected since fosfomycin is a small molecule. Its elimination half life ($T_{1/2}$) ranged from 1.2 to 1.6 hours. The serum and interstitial fluid levels parallel each other with maximum concentrations between 32 and 80 μ g/ml. Linear relationships between the area under the curve (AUC) values in serum and interstitial fluids were found suggesting the non-dose-dependent kinetics of fosfomycin. It also suggests that fosfomycin does not accumulate in body organs and tissues. This is in agreement with earlier findings [71].

The antimicrobial killing mode of action of fosfomycin has been well summarized [7, 43, 68, 72, 73] (see especially reference [73]). Fosfomycin (also fosmidomycin) enters the bacterial cell via the action of the permease systems that regularly transport L- α -glycerophosphate and D-glucose-6-phosphate (which also induces the hexose phosphate uptake system providing a second means for fosfomycin transport). Glucose-6-phosphate has been demonstrated to potentiate the therapeutic efficacy of fosfomycin in mice infected with a variety of strains of *E. coli* [74]. Once inside the bacterial cell, fosfomycin covalently bonds to phosphoenolpyruvate transferase inhibiting the enzymatic condensation of UDP-N-acetyl-glucosamine with phosphoenolpyruvate (phosphoenolpyruvate is a chemically analogous metabolite of fosfomycin), the first step of cell wall (murein) biosynthesis. This system is unique to bacterial cells and does not exist in mammalian cell [68] thereby giving the reason for the selective toxicity of fosfomycin. Fosfomycin is bactericidal only for proliferating microorganisms.

The mechanism of development of bacterial resistance to fosfomycin is rooted in its mode of action and transport [73, 74]. Mutations which cause bacteria to lose or possess poorly developed transport systems are the primary cause for the emergence of resistant strains. Thus, a chromosomally-mediated single-step mechanism of resistance can occur at high frequency in populations

of Gram-negative bacteria. In addition to the above-mentioned mechanism of resistance acquisition, it has been shown that plasmids and transposons were responsible for the transfer of resistance through drug modification and inactivation in *Serratia* strains and other Gram-negative bacilli [75-79].

A large number of in vitro studies and some studies in experimental animals and humans have been performed using fosfomycin in combination with other antibacterial agents in order to explore the possibility of enhancing its activity and to retard, or prevent, the emergence of resistant organisms. Enhancement of fosfomycin activity is especially important in serious infections where heavy bacterial populations can be a problem and emergence of resistant microorganisms can also become a therapeutic problem in many instances. The number of publications related to this area is too numerous to present in our list of references individually and we suggest one review article for the purpose of orientation and specific citations [80]. We will, instead, present here a list of those antibiotics which have been employed in combination with fosfomycin against a variety of bacterial strains. Among the agents used are beta-lactams (penicillin G, ampicillin, carbenicillin, methicillin, cephalothin, cephaloridine, cephalexin, cephradin, cefazolin, cephapirin, cefamandole, cefmetazole), aminoglycosides (streptomycin, gentamicin, kanamycin, tobramycin), chloramphenicol, vancomycin, tetracycline, rifampin, imipenem, sulfamethoxazole, trimethoprim and others. As expected, the observed results varied from no change to additive action to synergism and enhancement of activity. Since so many conditions and factors are present to influence the results of such in vitro studies, experts have regarded these data with great suspicion [43]. There are, nonetheless, several prospective double-blind clinical trials which have tested fosfomycin in combination with adequate and safe results. Among these studies are the combination of fosfomycin and metronidazole in elective colorectal surgery [81], fosfomycin in combination with amoxicillin ("Co-fosfolactamine") in the treatment of urinary tract infections [82] and the combination of fosfomycin and ampicillin or chloramphenicol in the treatment of typhoid [83].

Based upon its favorable clinical pharmacology [84, 85], fosfomycin has been widely used parenterally, as its calcium or lysine salt, mainly in Europe (Spain, Italy) and Japan. Initially its use was confined to the treatment of acute urinary tract infections [86-88]. Regular dosage was 2 g per day for several days with variable results against *E. coli*, *S. marcescens*, *P. vulgaris*, and perhaps, *S. aureus*. Oral fosfomycin (2 g daily) was found to be effective in the treatment of staphylococcal nasal carriers [89] but is not indicated in the therapy of gonococcal infections [90].

The bacteriology, clinical-pharmacology and clinical applications of fosfomycin are summarized in a very thorough monograph covering the area up to 1983 [91]. In this review, the use of fosfomycin is extended to pediatric infections.

Fosfomycin has also been administered, probably for better therapeutic effects, intravenously as its disodium salt which is a crystalline, white hygroscopic powder distributed in ampoules containing one or four grams. It is administered twice daily as an intravenous short-infusion (15–30 min) at a dose of 100–200 mg/kg body weight depending upon the causative agent and the severity of the infection. The daily dose varies and may be 2×2 g or 2×8 g or, occasionally, 3×8 g intravenously. The duration of treatment may be from 5 days to 3 weeks (generally 6–9 days). Three representative cases which were not included in the above-mentioned monograph [91] are briefly described below.

A patient with a severe hand infection with necrosis caused by *P. aeruginosa* and *Serratia* sp. was treated with fosfomycin (3×3 g daily) in the form of a short infusion for nine days with complete healing of the wound and disappearance of septic complications [92]. In another report [93], thirteen patients with otitis media and other infections of the middle ear and its region were treated with fosfomycin given as intravenous infusion for 4 to 9 days with the daily dose varying from 2×2 g to 2×8 g. The pathogens seen in the study were (with numbers of different strains in parentheses) *P. vulgaris* (6), *P. mirabilis* (1), *S. aureus* (5), *P. aeruginosa* (3) and streptococci (2). With the exception of one of the *P. vulgaris* strains, all responded as sensitive to fosfomycin with consequent therapeutic effects. In the third study [94], fosfomycin was administered to 19 patients as a single 8 g intravenous infusion over 30 min five hours prior to cataract surgery. The concentration of fosfomycin in the primary aqueous humor peaked at 35–60 $\mu\text{g/ml}$ 1–2 hours after the start of the infusion. This concentration appears to constitute an effective perioperative prophylaxis which can also serve as an initial therapy (8 g) for intraocular Gram-positive or Gram-negative infections.

In contrast to studies performed in mice, oral administration of the calcium or lysine salts of fosfomycin in humans results in poor and variable absorption. This kind of species difference is known to occur with many other drugs as well. Thus, oral administration of fosfomycin in humans does not regularly produce high enough protective blood and tissue levels of the drug. In the case of the calcium salt, blood levels reach only about 10 $\mu\text{g/ml}$ while intramuscular administration of 1–2 g of the disodium salt yields blood levels of 30–40 $\mu\text{g/ml}$ and intravenous administration reaches even higher blood levels.

This situation necessitated the search for salts of fosfomycin with better oral absorption. Among the many salts formed with organic bases (not published), the derivative of fosfomycin with tromethamine or trometamol [tris-(hydroxymethyl)-aminomethane] represents a substantial improvement in oral availability [95, 96].

Fosfomycin trometamol ("Monuril"), whose structure is shown in Fig. 6, is a highly water-soluble monobasic salt of fosfomycin. It has been demonstrat-

ed to be absorbed faster and more efficiently producing much higher and more prolonged serum levels and less individual variations than earlier developed salts of fosfomycin [97]. Because of its high and sustained blood levels, it proved to be an effective prophylactic agent in transurethral prostatic surgery in a controlled multicenter clinical trial when given as a single 3 g dose [98]. After oral administration of 2 g dosage, peak serum levels were about 21 $\mu\text{g/ml}$ and high concentrations (about 3000 $\mu\text{g/ml}$) were found to be excreted in the urine. Distribution of the drug in tissues also reached high levels. The safety profile of the drug appeared to be excellent with only mild, transient gastrointestinal disturbances reported. In children after a single oral dose 13–63 mg/kg, the peak serum levels of fosfomycin reached 22.5 $\mu\text{g/ml}$. The peak urine concentration was found to be 2900 $\mu\text{g/ml}$ and even after 36–48 hours following the original dose, the concentrations remained above the MICs. The drug exhibited very good results in the treatment of uncomplicated urinary tract infections without adverse side-effects. Some of the early detailed studies on fosfomycin can be found in the literature [99].

Recently, an interesting and important observation has suggested that fosfomycin can protect or diminish the nephrotoxic effects of aminoglycosides (such as gentamicin or amikacin) in experimental animals and humans [100, 101]. This effect has been shown to be protective but not curative since fosfomycin must be present at the site of action simultaneously with the aminoglycoside having similar pharmacokinetics. This unexpected finding, which is presently under critical study and evaluation, permits us to conclude that fosfomycin enjoys not only a very interesting present but also a broader and promising therapeutic future in our fight against infectious diseases.

Alafosfalin

Alafosfalin (formerly alaphosphin) is perhaps the most studied member of the phosphonic acid-containing phosphonopeptide family of compounds. It was designed, synthesized and developed on a rational basis as an inhibitor of a biochemical process unique to cell wall biosynthesis in procaryotic organisms [102]. Alafosfalin (L-alanyl-L-l-aminoethylphosphonic acid) is a small molecule, whose structure is shown in Fig. 6, having a molecular weight of 168. It is capable of inhibiting the growth of many Gram-negative pathogenic bacteria *in vitro* and also in experimentally infected laboratory animals.

Alafosfalin was selected for detailed study of its antibacterial activity from more than 300 phosphonopeptides synthesized on the basis of structure-activity relationships, especially the dipeptide compounds which mimic the structure of the terminal moiety (D-alanine-D-alanine) of the bacterial cell wall peptidoglycan [103, 104].

The in vitro antibacterial activity of alafosfalin should be established in defined media (as is the case for cycloserine, sulfonamides and fosfomycin) free from antagonists of small peptide mimetics. In addition to this, the MIC values obtained for alafosfalin are highly influenced by the type of medium (liquid or solid), pH (it appears to be more active at acidic than at neutral pH) and size of the test inoculum. Rapid development of resistant colonies has also been described. These are the reasons for the varied MIC values reported for the drug. Table VIII (adapted from reference 102) demonstrates the inoculum and pH effects on the MIC values of alafosfalin. It is more active against Gram-negative than against Gram-positive bacteria. Practically all pathogenic Gram-negative bacteria tested, with the exception of *P. aeruginosa* and *P. mirabilis*, are susceptible to the action of alafosfalin [105, 106]. It is more active against *E. coli* (MIC about 0.03 µg/ml) than against other genera such as *Klebsiella*, *Enterobacter* (MICs about 1.0 µg/ml) or *Serratia*, *Citrobacter*, *Salmonella*, *Shigella*, *Providencia* (MICs about 4.0 µg/ml). It has been shown to possess activity against the ampicillin resistant strains as well. Of the Gram-positive cocci, *S. faecalis* is as susceptible as the Gram-negative bacilli but *S. aureus* is much less susceptible.

Some modest bactericidal synergism or potentiation of activity has been observed between alafosfalin and other cell-wall active antibiotics such as cycloserine or the beta-lactams especially cephalixin [106–108].

Alafosfalin was shown to be active against anaerobic organisms such as *Bacteroides* spp. (MIC 0.5–25 µg/ml), *Fusobacterium nucleatum* (MIC 0.8 µg/ml) and *Clostridium perfringens* (MIC 12.5 µg/ml). Activity was not observed, however, against *Clostridium difficile* [109].

The fact that alafosfalin requires special, antagonist-free defined media for routine susceptibility determinations may be a drawback to its routine use in clinical situations.

Table VIII

Effect of pH and inoculum size on MIC values of alafosfalin in defined medium

Organism	Inoculum of 10 ⁸ c. f. u.*		Inoculum of 10 ⁷ c. f. u.	
	pH 5.5	pH 7.5	pH 5.5	pH 7.5
<i>Escherichia coli</i>	0.12	8	4	> 512
<i>Escherichia coli</i> (penicillin resistant)	0.03	1	1	128
<i>Klebsiella aerogenes</i>	0.06	8	2	256
<i>Shigella boydii</i>	0.003	2	2	8
<i>Serratia marcescens</i>	1	128	512	> 512
<i>Salmonella typhi-murium</i>	0.5	64	8	512
<i>Staphylococcus aureus</i>	8	128	> 512	> 512
<i>Staphylococcus faecalis</i>	0.25	2	2	8

* c. f. u. = colony forming units

Alafosfalin has been shown to be bactericidal for susceptible Gram-negative bacteria at the MIC or somewhat higher concentrations with rapid lysis of cells observed [102, 110, 111]. Figure 7 (adapted from ref. [110]) demonstrates that alafosfalin in antagonist-free medium, or the newly developed colourless peptone glucose buffered broth, can bring about lysis of *E. coli* strains in exponential growth after one hour at a concentration as low as 1.5 $\mu\text{g}/\text{ml}$. This lytic action has been demonstrated to be independent of beta-lactamase production. In *E. coli* which have developed resistance to alafosfalin (defined as regrowth after partial lysis), it was demonstrated that total lysis can be induced by co-

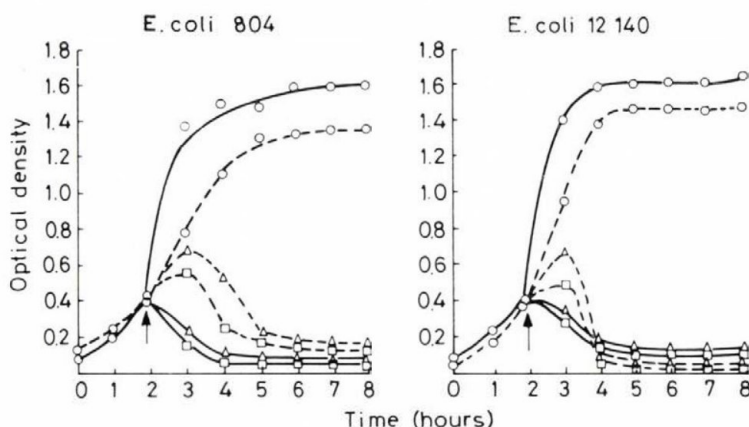


Fig. 7. Lytic action of alafosfalin on the strong beta-lactamase producing *E. coli* strain 804 (left) and on the non-beta-lactamase producing *E. coli* strain 12140 (right) in peptone-glucose-buffered broth and in supplemented antagonist-free medium. ——— antagonist-free medium; - - - - - peptone-glucose-buffered medium; ○ control; △ 1.5 $\mu\text{g}/\text{ml}$ and □ 3 $\mu\text{g}/\text{ml}$ alafosfalin; ↑ time of addition of alafosfalin to culture

administration of subinhibitory concentrations of ampicillin, suggesting a true in vitro synergism between the two cell wall active antibiotics. Against Gram-positive cocci, alafosfalin is non-lytic and only bacteriostatic.

The in vivo animal infection-protection experiments have reflected these in vitro results [102]. Studies in which mice were treated subcutaneously 1, 3 and 5 hours after challenge, demonstrated ED_{50} values of 5–10 mg/kg against *E. coli* and 25–50 mg/kg against *Klebsiella aerogenes*.

In rats, dogs, baboons and human volunteers, parenterally administered alafosfalin was shown to be absorbed quickly and dose-related serum levels achieved within twenty minutes. The elimination half-life ($T_{1/2}$) was about 10 min in mice and about one hour in humans and baboons. It was shown in rabbits that serum levels fell rapidly with a $T_{1/2}$ of 25 min after intravenous injection of 100 mg/kg. Maximum concentrations were reached in the tissue cage fluid 60–90 min after injection and slowly declined thereafter [112].

Alafosfalin was shown to be well absorbed after oral administration in rats, baboons and humans but extensive hydrolysis to alanine and L-1-aminoethyl-phosphonic acid was observed. This first-pass metabolism was less extensive in humans than in other animals [113, 114]. In humans, the serum levels after oral ingestion of 500 mg were shown to be similar to those levels obtained with intramuscular injection of 200 mg. The unchanged drug and metabolites can be found excreted in the urine in amounts equivalent to 6–17% (or 20–30%) of the original dose. Alafosfalin has a considerable margin of safety in animals and humans and the ongoing clinical trials will ultimately decide whether this drug can contribute significantly to our anti-infective armamentarium.

Alafosfalin inhibits the biosynthesis of various bacterial cell walls [115–117]. In its mechanism of action, three steps appear to be involved. First, an active transport of the drug by a peptide permease. Second, once inside the bacterial cell, peptidase cleavage to L-alanine and L-1-aminoethylphosphonic acid. Third, inhibition of two enzymes involved in the early stages of peptidoglycan synthesis, namely alanine racemase and L-alanyl-uridine-diphosphate-muramyl-ligase. In addition to the above-mentioned steps, it can also act as a false substrate resulting in the prevention of peptide chain extension and disruption of the cell wall with ensuing cell lysis.

Fosmidomycin

Fosmidomycin (FR-31564), chosen for development among four phosphonic acid compounds, is the most recent member of the phosphonic acid-containing antibiotics [118, 119]. All of the four compounds were of *Streptomyces* origin, closely related structurally, acidic white crystalline products, variably active in vitro and in vivo, capable of producing spheroplasts of susceptible bacterial cells and remarkably non-toxic to mice. FR-31564 was found to be the most active against Gram-negative organisms and was selected for further study under the name of fosmidomycin.

Fosmidomycin is produced by *Streptomyces lavendulae* [120]. It was isolated from the fermentation broth [121] and its chemical structure (shown in Fig. 6) determined [122]. It is a colourless, crystalline material determined to be 3-(*N*-formyl-*N*-hydroxy-amino)-propylphosphonate as a monosodium salt with a molecular weight of 183.

Fosmidomycin is active in vitro only against Gram-negative bacteria. Among the strains sensitive are *P. aeruginosa* (but not *P. maltophilia* or *P. cepacia*), *E. coli*, *Enterobacter* sp., *K. pneumoniae*, *Salmonella*, *Shigella* and *P. mirabilis* but not indole-positive *Proteus* strains or *Serratia* sp. It has also been shown to be active against strains resistant to other antibiotics and some anaerobic species. Gram-positive bacteria have been shown to be insensitive to fosmidomycin [121, 123, 124].

The type of medium (nutrient agar is most favourable), pH and inoculum size greatly influence the in vitro activity of fosmidomycin. A ten- to twenty-fold dilution of the nutrient broth or agar has been shown to further increase the sensitivity of the assay two to four-fold [125]. Addition of blood (but not human serum), glucose-6-phosphate or fructose-6-phosphate to the medium has also been shown to increase the activity of fosmidomycin. The in vitro action of fosmidomycin has been shown to be bactericidal.

Fosmidomycin was shown to be more effective in mouse infection-protection assays than would be expected from the observed MIC values obtained in vitro [123, 124]. The subcutaneous ED₅₀ values were found to be below 1 mg/kg for infections caused by *K. pneumoniae*, *P. vulgaris*, *P. rettgeri* and *Citrobacter freundii*; between 1 and 2 mg/kg for infections caused by *E. coli* and *P. aeruginosa*; 6 mg/kg against *P. mirabilis*; 8 mg/kg for infections caused by *E. aerogenes* and *E. cloacae*; and 37 mg/kg against infections caused by *Morganella morganii* [123]. Another study found ED₅₀ values of 11 mg/kg against *P. aeruginosa* and 2.7 mg/kg against *E. coli* when the drug was administered orally [124]. ED₅₀ values for subcutaneous administration of fosmidomycin in this study were found to be 14 mg/kg against *P. aeruginosa* and 3.2 mg/kg against *E. coli*. ED₅₀ values of 2.5–7.2 mg/kg for subcutaneous administration of fosmidomycin against *P. aeruginosa* compared to 56 and 100 mg/kg for fosfomycin have been reported [125]. Other investigators have also found fosfomycin less effective both in vivo and in vitro than fosmidomycin.

The mechanism of action of fosmidomycin is similar to that of fosfomycin, namely the inhibition of phosphoenolpyruvate transferase which is a very early step in the biosynthesis of bacterial cell walls [68, 95]. There appears to be a cross-resistance between fosfomycin and fosmidomycin. The active transport systems are common to both antibiotics and resistant mutants of *P. aeruginosa* have been shown to lack the L- α -glycerophosphate transport system [126].

Fosmidomycin interacts synergistically with beta-lactam and aminoglycoside antibiotics in studies using *Pseudomonas* and *Enterobacteriaceae* isolates [127].

Fosmidomycin possesses good pharmacokinetics [128]. It seems to be better in mice after oral administration. In dogs, the T_{1/2} was found to be 1.14 h, and peak serum concentrations of 41 μ g/ml and 16.6 μ g/ml were obtained after an intramuscular dose of 20 mg/kg and an oral dose of 40 mg/kg, respectively. In volunteers, peak serum levels were found to be 2.45 μ g/ml after an oral dose of 500 mg/kg suggesting unsatisfactory absorption. However, parenteral administration of the drug presents a better picture in humans with serum levels reaching 157 μ g/ml after intravenous injection and 12.3 μ g/ml following intramuscular injection. Urinary elimination of the drug is about 40% and it may be found excreted primarily in the unchanged form. Tissue and body fluid dis-

tribution is good and serum protein binding very low (4% or less in mouse, rat, dog and human studies). In volunteers, no adverse reactions or laboratory abnormalities were observed following intravenous administration of fosmidomycin.

On the basis of these highly favourable pre-clinical data, fosmidomycin continues to be developed in both oral and parenteral forms and awaits further clinical trials. It has already passed the phase I tolerance studies [129] and the human pharmacokinetic and preliminary phase II/A clinical studies [130] and has been recommended for further clinical evaluation.

Monophosphams and phosphonic cephalosporins

During the ambitious screening programs of the last decade for beta-lactam agents which are more specific, potent and stable to the actions of beta-lactamases and which possess increased pharmacokinetic properties with different and unusual spectra, the inclusion of phosphorus, especially phosphonic acid residues, into the basic nuclei of the beta-lactam structures, was quite naturally inevitable.

All of the compounds to be mentioned in this section are those which have been less-studied and for which there are only preliminary published data or personal communications available.

In the area of monocyclic beta-lactam antibiotics, a series of monophosphams have been synthesized and studied [131]. In these compounds, the phosphonic acid, or substituted phosphonic acid replaces the *N*-sulfonic acid group of the monobactam nucleus to which, at the 3-amino position, either aminothiazole or alkoxyaminothiazole side chains are attached. The *in vitro* antibacterial activities of these compounds were compared to those of their monobactam counterparts. These new compounds possess various types of spectra. Usually they have only Gram-negative activity, less activity against Gram-positive organisms and less anti-pseudomonal activity. The inherent beta-lactamase stability of the monobactams was not altered in these monophosphams.

The synthesis, without biological evaluation, of *N*-phosphorylated mono- and bicyclic beta-lactams has been reported [132].

Three mandelic acid-type (SKF 81151, 81373 and 83540) and one aminothiazolemethoxime-type (SKF 88082) cephalosporins with tetrazolemethyolphosphonic acid or ethyl-substituted phosphonic acid side chains at the 3-position of the cephem nucleus have been synthesized and tested *in vitro*. These compounds have been shown to have good, but not spectacular, spectra of *in vitro* activity similar to their non-phosphorus-containing counterparts [133]. There are most likely many more such compounds buried in laboratory notebooks awaiting eventual detailed evaluation.

An interesting paper has reported the synthesis of 1-phosphocephalosporins. In these compounds, phosphorus replaces the sulfur atom in the 7-amino-cephalosporanic acid nucleus. No biological data are available concerning these compounds [134]. In another report, the synthesis of phosphonic acid and phosphonic acid analogs of penicillamine, without biological data, was announced [135]. It is expected that the list of phosphorus-containing beta-lactam antibiotics will continue to grow.

Other than antibacterial phosphorus/phosphonic acid-containing compounds

In addition to the antibacterial/antifungal phosphorus-containing drug substances and drug products previously discussed, there are compounds within this group with antiviral, antitumour, antiprotozoal and various other pharmacological activities and efficacies. Space will not permit us to review and describe these chemical entities in the same manner as we have for the antibacterial and antifungal phosphorus/phosphonic acid-containing antibiotics. Instead, we will list and highlight here some of the newer and more interesting agents among them. This will be done so that we may be able to close our circle of discussion without creating a feeling of uncompletedness.

Of the antiviral phosphonic acid compounds, phosphonoacetate and phosphonoformate are attracting more interest and importance. These small, simple molecules are weakly active but very non-toxic antiherpetic agents with favourable pharmacological properties. They interfere with the virus-specific DNA polymerase at a pyrophosphate binding site. They have been shown to selectively inhibit the replication of herpes simplex virus types 1 and 2. Human trials have provided encouraging results in the treatment of herpes labialis [136]. Among more than 100 congeners, none was better than phosphonoacetic acid against herpesviruses *in vitro*. The intactness of the carboxylic and the phosphono groups and the distance between them appear to be important aspects in the antiviral activity of these agents [137].

Phosphonoformic acid, or trisodium phosphonoformate (Foscarnet) in addition to the above-mentioned effects, also inhibits the reverse transcriptase of human immunodeficiency virus (HIV). Its clinical evaluation is in progress [138]. These compounds can be used, oftentimes synergistically, with other antiviral agents.

Two new cyclophosphamide isomers appeared recently on the cancer chemotherapy horizon. They are Isofosfamide [139] and Mafosfamide [140]. The former of these has shown promise in the treatment of resistant testicular tumours and renal cell carcinoma, and the latter in the treatment of myeloid leukaemia. Another agent, Fosfesterol, is currently being studied for use in the treatment of ovarian tumours. These agents appear to be less toxic and more

effective when administered fractionally than in a single push injection at the same total daily dosages [141].

The sodium salt of dichloromethylene-bisphosphonic acid (Clodronate) is recommended for the treatment of osteolytic bone metastases and as an adjuvant therapy in malignant hypercalcaemia.

A derivative of fosfomycin, synthesized by attaching another phosphonic acid group to the original phosphonic acid group, has been found to be effective against *Schistosoma mansoni* and *Biomphalaria pfeifferi* [142].

A phosphorus-containing inhibitor of angiotensin I converting enzyme (ACE) has been isolated as a fermentation product of a species of *Actinomadura*. It is a white powder which is soluble in alkaline water. Its acid hydrolysis yields tyrosine, *N*-methylalanine and 1-amino-2-(4-hydroxyphenyl)ethylphosphonic acid. It is a potent ACE inhibitor with an activity somewhat less than that of captopril [143].

On the basis of a structural analogy to acetylsalicylic acid (aspirin), 2-phosphonoxybenzoic acid (Fosfosal) has been synthesized and studied as a new salicylate derivative. It is water soluble (in contrast to aspirin), possesses analgesic, anti-inflammatory and antipyretic effects with no irritative effects which can be damaging to the gastric mucosa. It does not inhibit prostaglandin biosynthesis, but it has been shown to be a strong inhibitor of cyclic AMP-phosphodiesterase [144].

There are many other phosphonic acid/phosphorus-containing compounds in addition to those having antimicrobial actions and discussed above. Many of these are important biocatalyzators, complexing agents, insecticides, herbicides, nerve gases, fertilizers, detergents, enzyme inhibitors and others [135]. The versatility of their interesting and practical uses in every day life is encountered in a newly published book [145].

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THE KINETICS OF CELL DIVISION IN A SYNCHRONIZING FERMENTOR

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The kinetics of cell proliferation of *Candida tropicalis* synchronized by periodical glucose starvation was examined in a continuous synchronizing fermentor. The length of the deterministic (B) and stochastic (A) phase of the cell cycle was determined by cell density data analysis using the Transition Probability Model. The size distribution of the population shows that the absence of nutrient stops the cells at the beginning of the cell cycle but the succeeding cell divisions are not perfectly synchronized. The reason of this comes from the difference between the generation times of the cells which is the consequence of the different long "A" phases in their cycle.

Synchronization of the cell division, a fundamental method in the study of the cell division cycle [1, 2], can be achieved in two different manners, namely induction and selection. The simplest way of induction synchrony is transferring starved cells into fresh medium [3]. This method can easily be done continuously in very different ways [4–6].

It is known from the genetic analysis done in Hartwell's laboratory that *Saccharomyces cerevisiae* cells starved for an essential nutrient are arrested at or before the *cdc28* mediated — namely "start" — step of the cycle [7, 8]. This is because cells cannot satisfy the growth-related requirements of the start in the absence of a given nutrient [9]. Thus, these cells initiate a new cycle synchronously after being transferred into a fresh medium. However, this does not involve that the cells will divide synchronously too, because their generation times are not equal [10]. The main aim of this paper is to analyze the cell division in a synchronizing fermentor by the Transition Probability Model of the cell cycle describing the generation time distribution [11].

Materials and methods

Yeast strain, media and culture conditions. *Candida tropicalis* strain NIH-21 obtained from Dr. E. K. Novák (National Institute of Hygiene, Budapest) was used throughout this study. The shaken cultures were incubated in Erlenmeyer flasks with vigorous rotary shaking in a water bath at 30 °C. YEPG medium of the following composition was used for growth of the organisms: glucose, 2 g; peptone, 1 g; yeast extract, 1 g; distilled water, 100 ml.

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Continuous cell synchronization. The equipment used for continuous synchronization for *C. tropicalis* cells (Fig. 1), is in principle the same as that used by Dawson [12, 13]. It is based on the synchronizing effect of nutrient starvation. Contrary to the continuous chemostat fermentation the feeding is periodical. This is assured by the upper feeding tank, into which the following nutrients were dripped with a constant rate: glucose, 5 g; $(\text{NH}_4)_2\text{SO}_4$, 3 g; KH_2PO_4 , 3 g; yeast extract, 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.25 g; distilled water, 1 litre.

The rate was set by the dripping and the height of the syphon fitted on the feeding tank, so that in every 4 h 20 ml medium was collected in the tank, which by floating into the synchronizing fermentor decreased the cell density of the 20 ml growing yeast suspension to its half. One half of this diluted culture — after being mixed by air bubbles with the help of the syphon — moved into the analyzed fermentor, which worked under the same conditions as the previous one. This culture was sampled for the measurements.

The fermentors and the feeding tank were thermostated at 30 °C. The whole apparatus was sterilized with ethanol (70 g/litre) before the experiment.

Determination of cell number and cell volume distribution. The cell density and the stage in the cell cycle of the yeast cells were followed by an electronic particle counter system (PSL-1, Medicor, Hungary) with a 70 μm^3 aperture, connected to an amplitude analyzer (PSA-1, Medicor, Hungary). The cell suspension was diluted before the determination with NaCl solution (9 g/litre) previously filtered through a 0.2 μm pore diameter membrane filter (Gelman).

Determination of glucose. The glucose concentration in the growth medium was determined by a glucose-sensitive membrane electrode containing immobilized glucose-oxidase (Radelkis, Hungary) using the standard addition technique.

Results

Cell density and glucose concentration changes in the synchronizing apparatus during the first 5 cycles after the inoculation of the system with a shaken culture are shown in Fig. 2. Despite of the fact that in the first 3 cycles the cycle period of the culture was not exactly 4 h, its approaching the stationary state could be followed very well. The linearity of cell density data to time on semi-logarithmic plot refers to an asynchronous multiplication. The generation time was slightly increasing with the number of cycles which is indicated by the flatter and flatter lines.

The repeated increase of cell density at the beginning of the cycles is explained by the fact that the cells could not exhaust all the glucose from the medium, consequently they multiplied exponentially during the whole cycle with a generation time shorter than the cycle time of the apparatus. Dilution to the double at the end of the cycle could not reestablish the starting density of the previous cycle as the growth was more than two-fold during the cycle. Therefore if glucose is not totally consumed in the n -th cycle, then the cell density at the beginning of the $(n+1)$ -th cycle will change according to

$$N_{n+1} = \frac{N_n \cdot 2^{T/t_g}}{2} = N_n \cdot 2^{T/t_g - 1}$$

where T (the cycle period of the apparatus) and t_g (the generation time) are higher than in the beginning of the n -th cycle (N_n), as $T/t_g > 1$ thus $2^{T/t_g}$ is also more than 1.

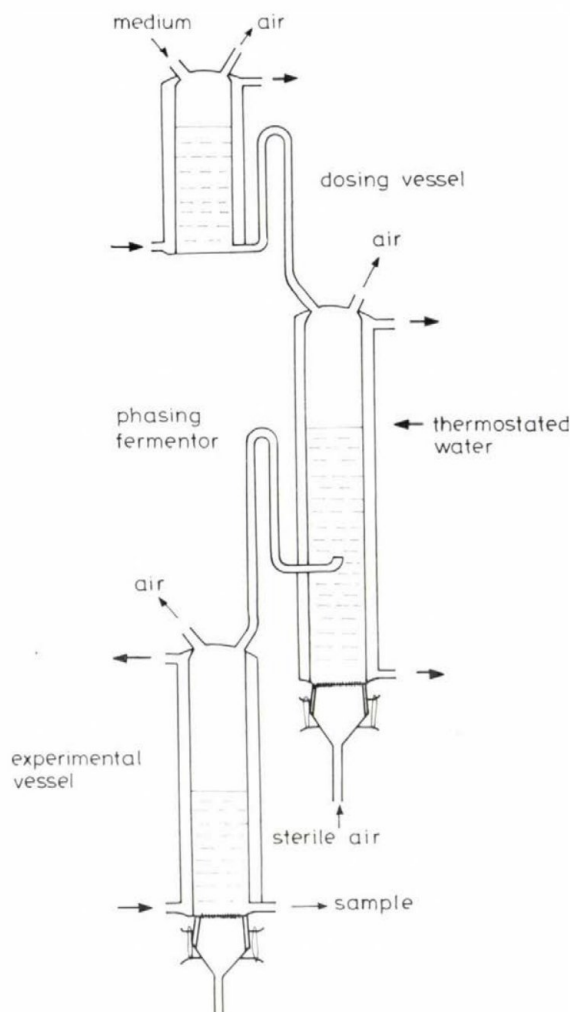


Fig. 1. The continuous synchronizing fermentor

The following equation describes the relationship between the glucose concentration ($G1_{n+1}$) at the beginning of ($n+1$)-th cycle and glucose concentration at the end of n -th cycle ($G1_n$):

$$G1_{n+1} = \frac{G1_n + 0.5}{2}$$

as the medium contains 0.5% glucose and is mixed to the cell suspension in 1 : 1 ratio at the end of cycle.

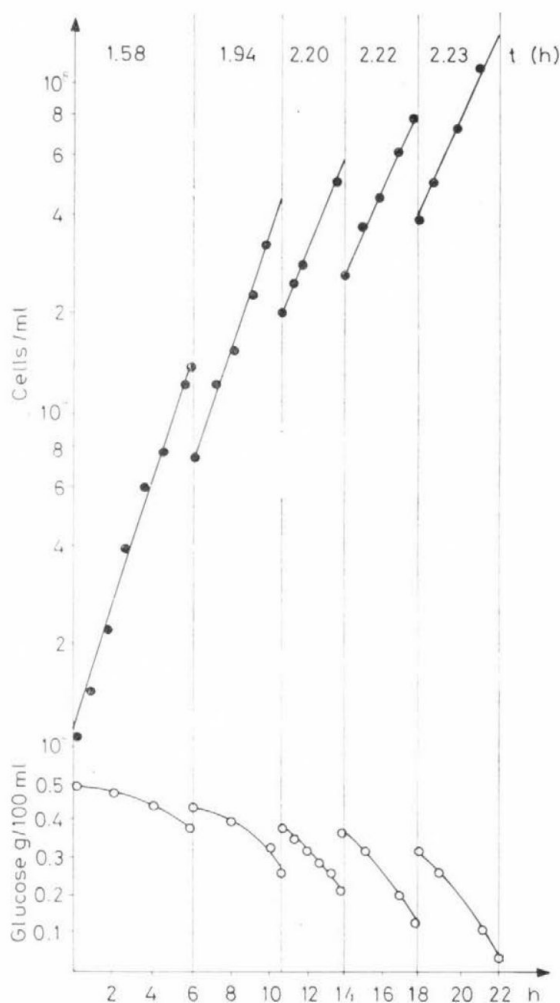


Fig. 2. The change of cell density and glucose concentration during asynchronous growth

Since the cell suspension uses glucose always more quickly, the glucose concentration at the beginning and at the end of the individual cycles will be lower and lower (see Fig. 2). This process lasts until the glucose will have been consumed in one of the cycles (the 5th cycle represents this condition most closely) and from this point on the glucose concentration will always be 0.25% at the start of the cycle. After this point the cell density at the beginning of the cycles must become constant at a level which allows for all cells to divide only once on the initially present 0.25% glucose. This steady-state of the fermentor is shown in Fig. 3, where the four cycles are exactly the same concerning the

beginning and the final density and concentrations. The logarithmic scale for cell density data serves only to help the comparison with the previous figure. The cell density changes significantly differ from the linear course characteristic of an asynchronous population. In the first hour of the cycle there is no increase in cell density and in the following two hours the cell number doubles while the glucose concentration gradually decreases from the initial 0.25% and reaches zero at about the third hour.

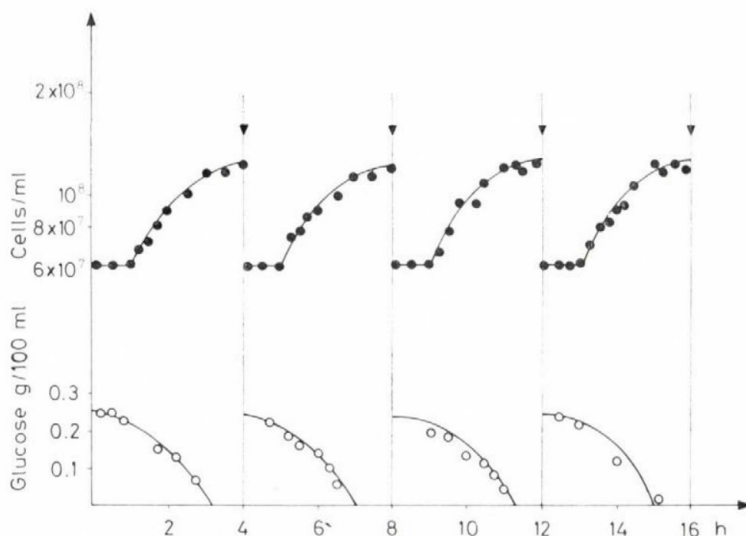


Fig. 3. The change of cell density and glucose concentration during synchronous growth

In Fig. 4/A the changes in cell density are based on the average of three 4 h cycles. The cell divisions cannot be considered perfectly synchronous since as long as about two hours have passed between the first and the last divisions. Perfectly synchronous cell division cannot be expected even from cells being initially in the same phase of the cell cycle because of the variability in the cell cycle phases.

Supposing that in the G_1 phase of *C. tropicalis* cell cycle there is a similar state to the start event of *S. cerevisiae*, the nutrient starved cells must stop before this step. The start was proven to be the rate-limiting step of the cycle, by which cells are passing into the practically definite long B period which is followed by the cell division, according to first order kinetics [14]. Consequently the logarithm of the percentage of undivided cells (α) will decrease linearly in time after the period B [11].

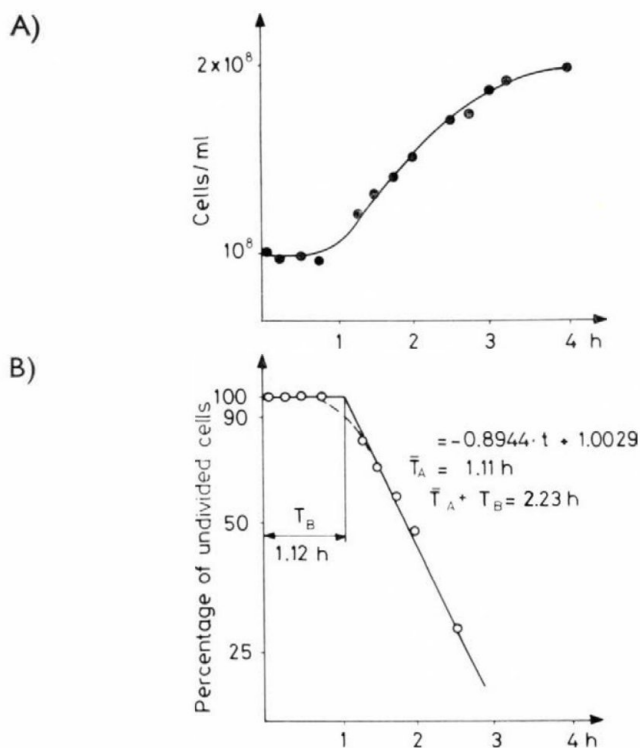


Fig. 4. Time course of cell density (A) and percentage of undivided cells (B)

Knowing the starting cell density (N_0), the percentage of undivided cells can be calculated at a given time with the following formula:

$$\frac{2N_0 - N}{N_0} 100 = \left(2 - \frac{N}{N_0} \right) 100$$

where N is the cell density at t time.

The so called " α " function calculated from the measured values is shown in Fig. 4/B. The points calculated from the experimental data fit well to the α -function predicted from the Transition Probability Model. Fitting a straight line with linear regression to the descending part of the curve we obtain the equation $\alpha = -0.0924 t + 1.0029$ from which the length of B phase is $T_B = 1.003/0.894 = 1.12$ h. If the number of undivided cells after T_B decreases exponentially following the equation

$$N_0 e^{-\lambda \cdot (t - T_B)}$$

where $-\lambda$ is the slope of the line (-0.8944) shown in Fig. 4/B, then the number of the divided cells changes according to

$$N_0(1 - e^{-\lambda \cdot (t - T_B)})$$

Since the number of the divided cells follows the running integral of the generation time distribution, derivating this equation we find that the generation time changes in $T_B < t < \infty$ interval according to the exponential distribution

$$f(t_g) = \lambda e^{-\lambda \cdot (t - T_B)}$$

The mean of this distribution equals to λ^{-1} which is the average period of the cells A phase. Adding this to T_B the mean generation time distribution of population is

$$T_B + \lambda^{-1} = 2.23 \text{ h}$$

which agrees well with the duplication time in the asynchronous population having the same density. Summarizing, it may be concluded that the extension of cell division times in such a wide interval comes from the variability of the cell generation times.

The analysis of cell volume distribution also helps investigating the population's synchrony. In an exponentially increasing asynchronous culture the cell volume distribution is constant all the time [15], since the increase in cell volume caused by cell growth is compensated by the decrease in volume caused by cell divisions. But if growing cells are collected at a certain point of the cell cycle and they start to grow again then the volume distribution changes typically with time [15].

The volume distribution of *C. tropicalis* cells during a 4 h cycle is shown in Fig. 5. The arrows indicate the most common cell volume of the given sample. The peak of the distribution curve in 15 min sample is at $21.2 \mu\text{m}^3$ and during the first hour it moves to the right towards larger volumes. This lasts until it reaches the $31 \mu\text{m}^3$ size (105 min) when a new peak appears at $16.3 \mu\text{m}^3$. Considering this, about $31 \mu\text{m}^3$ corresponds to the average volume of dividing cells, while $16.3 \mu\text{m}^3$ to that of the newborn cells. From the 105th min to the 165th the distribution is bimodal, both dividing and divided cells are present, but as the number of divided cells constantly rises, that of the dividing ones decreases and from the 195th min there are only descendents in the sample. The unimodal distribution characteristic of the recorded distribution at the beginning and at the end of the cycle also suggests that the disappearing of glucose stops most of the cells at the same phase in the cell cycle. In spite of this, the cell divisions are not synchronous since the generation times are not equal, which is confirmed by the bimodal distribution.

The change in the cell volume distributions during the cycle can be demonstrated by the change in the average cell volume (V), too. This was calculated with the formula

$$V = \frac{\sum N_i \cdot X_i}{\sum N_i}$$

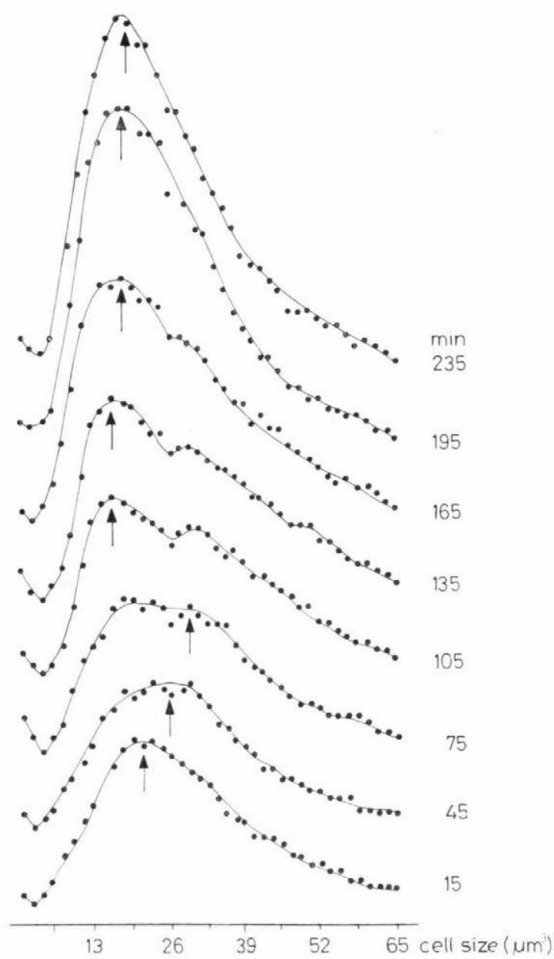


Fig. 5. Time course of the change of cell size distribution

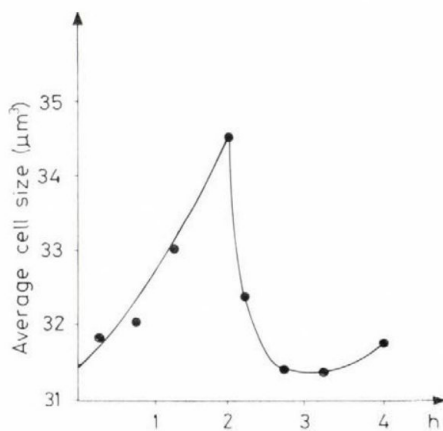


Fig. 6. Time course of the change of average cell size

where N_i is the number of cells and X_i the cell volume in the i channel ($0 < i < 64$): $X_i = 1.63 \cdot i$.

The changes in the average cell volume during a 4 h cycle are shown in Fig. 6. The increase of cell volume observed in the first two hours indicates that during this time the volume increase associated with cell growth is more than the volume decrease caused by divisions. Thus the rapid decrease in cell volume after the second hour suggests very intensive divisions.

Discussion

Smith and Martin[11] has proposed a Transition Probability Model of the cell cycle. In this model, the first part of the cycle, including most of G_1 , consists of the A phase, which is of indeterminate length. Each cell in the A phase has a constant probability (P) of leaving this phase in any time interval, when it enters the B phase. The duration of B phase (T_B), ending in mitosis, is essentially constant.

Our experimental data fit very well to the " α " function predicted from the Transition Probability Model. However, it does not mean that the cell cycle is simply controlled by a random cell cycle event. There is a substantial amount of evidence that the cell size controls the cycle time of growing cells [16], but in the Transition Probability Model there is no correlation between cell size and cycle time. However, a pure cell size control model is also inadequate to describe the cycle time distribution because it is deterministic. Therefore it seems adequate to put forward the hypothesis that the size control is sloppy and it gives a variation around the mean value. This so called sloppy size control model [17] gives a more adequate description for cell size and cycle time distribution of microbial populations than the Transition Probability Model does, but it also needs a more sophisticated mathematical analysis of experimental data.

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CHANGES OF ALTERNATIVE RESPIRATION DURING THE DIVISION CYCLE OF *CANDIDA TROPICALIS*

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The changes in cyanide-resistant and SHAM-sensitive respiration have been examined in the cell cycle of yeast cells synchronized by nutrient starvation. The changing of the cyanide-resistant respiration of cells proliferating in synchronizing fermentor, where the glucose concentration was continuously decreasing, was associated with the stage in cell cycle rather than with the alteration of environment. The cyanide-resistant alternative respiration may be attributed characteristic of a certain part of the cell cycle termed "A" phase. This hypothesis is supported by the observation that in cultures proliferating synchronously under constant conditions, the cyanide-resistant respiration changes periodically and reaches its maximum in the "A" phase after the cell division.

A great diversity of cell type, from bacteria to higher eukaryotic cells, exhibits some capacity for respiration even when inhibitors of the main phosphorylating respiratory chain are present at concentrations adequate to prevent electron transport by this route [1, 2]. In eukaryotes, the best known example of cyanide-resistant, so called "alternative" respiration involves mitochondrial systems [3, 4]. They are believed to consist of an electron transport chain comprising the first NADH-CoQ span of the normal respiratory chain and an unidentified terminal oxidase step transporting the electrons from CoQ to O₂ [1, 4]. This alternative CoQ-O₂ step is specifically inhibited by hydroxamates such as SHAM [5] and is not coupled to phosphorylation [4].

Several reports dealing with cyanide-resistant respiration in yeasts have appeared in the literature [6–8]. The degree of resistance varies widely upon the species used from a few percent insensitivity as observed in *Saccharomyces cerevisiae* to a marked stimulation as observed in *Candida* spp. by Hermann [7]. Moreover, Goffeau and Crosby [9] have distinguished between two types of antimycin- and also cyanide-insensitive respiration of the yeasts. They concluded that *S. cerevisiae* and *Schizosaccharomyces pombe* have a cyanide-resistant terminal oxidase system insensitive to hydroxamates, but sensitive to high concentrations of azide. Thus cyanide-insensitive respiration of these species being sensitive to hydroxamates, is significantly different from that of the candidas described.

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As the physiological function of the alternative respiration is still unknown [1, 2] and our main topic is the study of cell cycle regulation, the purpose of this work was to study the role of cyanide-resistant respiration in this process.

Materials and methods

Yeast strain, media and culture condition. *Candida tropicalis* strain NIH-21 obtained from Dr. E. K. Novák (National Institute of Hygiene, Budapest) was used throughout this study. The shaken cultures were incubated in Erlenmeyer flasks with vigorous rotary shaking in a water bath at 30 °C. YEPG medium of the following composition was used for growth of the organism: glucose, 2 g; peptone, 1 g; yeast-extract, 1 g; in 100 ml distilled water.

Continuous cell synchronization. The equipment used for continuous synchronization of *C. tropicalis* cells was described earlier [10]. It is based on the synchronizing effect of nutrient starvation and, contrary to the continuous chemostat fermentation, the feeding is periodical. The limiting nutrient was glucose and the cycle time of our apparatus was set to 4 h.

Measurement of oxygen uptake. Oxygen uptake was measured by a polarographic method in which a Clark type oxygen electrode was utilized. The respiratory measurements were carried out in a thermostated (30 °C) 10 ml volume vessel. The yeast suspension diluted by 0.05 M KH_2PO_4 -NaOH (pH 7) buffer was put in the reaction vessel. The electrode was fit into the reaction chamber so as to leave no air bubbles in the vessel. Inhibition of the respiration with SHAM and KCN were done as described by Bahr and Bonner [11], who expressed the total respiration (V_T) as

$$V_T = p \cdot V_{\text{alt}} + V_{\text{cyt}}$$

where V_{cyt} is the contribution by the cytochromic path, V_{alt} is the maximal capacity of the alternative path, "p", a number between 0 and 1, defines the fraction of the full alternative path which is operating.

The values of V_{alt} were experimentally obtained by the inhibition with SHAM in the presence of KCN. The operating alternative respiration ($v_{\text{alt}} = p \cdot V_{\text{alt}}$) was determined by the inhibition of respiration with SHAM in the absence of KCN.

Results and discussion

In Fig. 1/A the change of cell density is shown based on the average data of three 4-hour-long cycles. According to the "Transition Probability Model" of the cell cycle, the logarithm of the percentage of the non-divided cells (α) decreases in time linearly as shown in Fig. 1/B after the period B (S, G_2 , M and a part of G_1) which is the deterministic part of the cell cycle.

Figure 2 shows how the total and cyanide-resistant respiration of the suspension, proliferating in the analyzed fermentor, changes during a 4-hour-cycle. The appearance of a maximum in the total respiration can be explained as follows. The increase of the respiration which, being sensitive to cyanide, is cytochromic in nature, must be a result of the synthesis of cytochromes. At the beginning of the cycle, where the concentration of glucose is high, the available respiratory capacity of the cells works with maximal efficiency and, accordingly, an increase in the respiration is possible only by extending the available capacity. The decrease in the respiration rate after the maximum might be the consequence of the glucose concentration falling below a critical value.

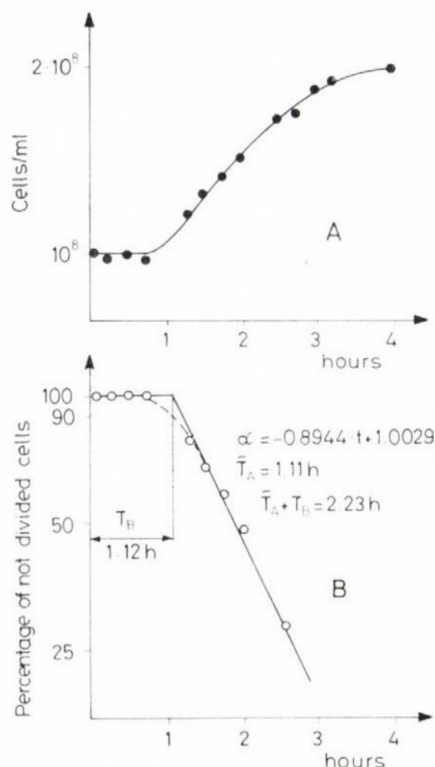


Fig. 1. Time course of the change in cell density (A) and percentage of not divided cells (B)

The extent of cyanide-resistant respiration doubles during the cycle and since the cell number increases also like that, the rate per cell is the same at the end and at the beginning of the period. However, the changing of cyanide-resistant respiration is not monotonous: first it decreases, then during the next hour it is not measurable and afterwards it increases continuously until it reaches the final value. The fact that with 2 mM SHAM it was always possible to reduce to zero the respiration resistant to 0.1 mM KCN, also proves that this cyanide insensitive oxygen uptake is a result of SHAM sensitive alternative oxidase activity.

We examined the effect of 2 mM SHAM to the total respiration of the cell suspension in the absence of KCN. Figure 2 also shows the effect of SHAM and KCN used together and alone on the total respiration of the cells at the beginning and at the end of the period. At the beginning, the participation of alternative respiration in the total O_2 uptake of the cells is $15 \text{ nmol } O_2 \text{ min}^{-1} \text{ ml}^{-1}$ which, being exactly equal to the available maximum capacity, indicates a total function. However, at the end of the period, the capacity of cyanide-resis-

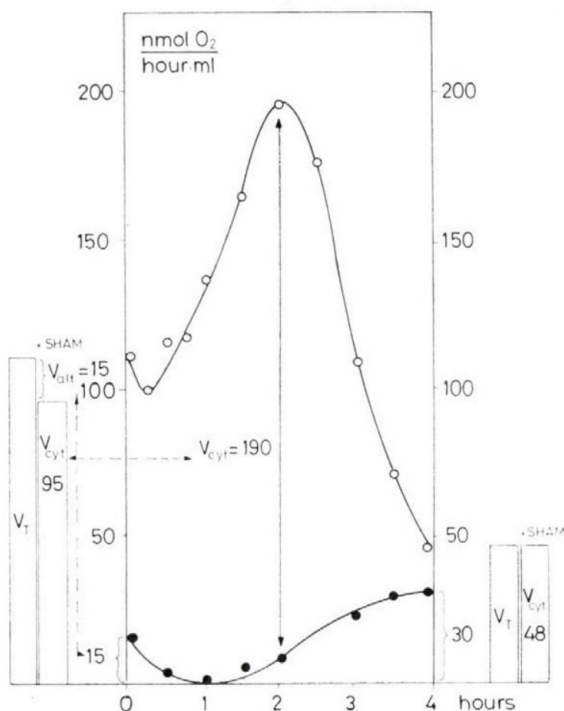


Fig. 2. Time course of changes in total (○—○) and cyanide-resistant (●—●) respiration

tant route is the double ($30 \text{ nmol O}_2 \text{ min}^{-1} \text{ ml}^{-1}$) as that of the beginning. As SHAM has no effect on the total respiration in the absence of KCN, there are two possibilities: the alternative oxidases either do not function normally, therefore SHAM does not inhibit the total respiration or they function, but diverts the electrons to the cytochrome pathway. On the basis of our present knowledge the first version is more likely [12].

The capacity of cytochrome respiration is about the double at the beginning ($95 \text{ nmol O}_2 \text{ min}^{-1} \text{ ml}^{-1}$) as that at the end ($48 \text{ nmol O}_2 \text{ min}^{-1} \text{ ml}^{-1}$) of the period. As it is seen in Fig. 2, in the second hour of the period this parameter reaches the $190 \text{ nmol O}_2 \text{ min}^{-1} \text{ ml}^{-1}$ value. Regarding the cytochrome capacity measured at the beginning of the cycle, we have already proven that it equals to the maximum capacity; the question arises now as to the end of the period. If it turns out to be less than the maximum value, then probably the lack of glucose is the reason for the lower activity of cytochromes in the presence of an alternative oxidase inhibitor. The second statement can be proven with a simple calculation. The starting parameters of the steady-state fermentor come from

the final ones by dividing them by two. By this adding the half of the apparent cytochrome capacity ($24 \text{ nmol O}_2 \text{ min}^{-1} \text{ ml}^{-1}$) to the half of the cyanide-insensitive respiration ($15 \text{ nmol O}_2 \text{ min}^{-1} \text{ ml}^{-1}$), we get only $39 \text{ nmol O}_2 \text{ min}^{-1} \text{ ml}^{-1}$ for the total respiration, which is less than the half of the measured value. However, calculating with the half of the highest cytochrome activity ($95 \text{ nmol O}_2 \text{ min}^{-1} \text{ ml}^{-1}$) measured during the period, the result reflects exactly the beginning total respiration ($110 \text{ nmol O}_2 \text{ min}^{-1} \text{ ml}^{-1}$). The above calculation has an other consequence too, since it helps to find the exact time of the cytochrome synthesis in the cell cycle. This process takes place in the first two hours, when the cytochrome respiration increases to its double (from 110–15 to 190). We should not have to count with cytochrome synthesis in the last two hours to explain the cytochrome activities at the beginning of the period.

The periodical change in alternative respiration is not a surprising phenomenon, since these kinds of change have been observed often in enzyme activities in the cell cycle of eukariotes [13]. The question is rather that this phenomenon is connected with the changes of the cell's stage during the cell cycle or it is simply due to the changing conditions (e. g. decreasing glucose concentration). A possible explanation for the observed changes is that the activity of alternative oxidases is repressed by glucose or is induced by high oxygen concentration. It is obvious that the level of dissolved oxygen in the medium changes exactly inversely to the total respiration and can run parallel to the changes in alternative respiration. However, the total respiration decreases only after the second hour and the activity of alternative oxidases increases after the first hour, at the time when the cell division starts. Therefore, we believe that the development of cyanide-resistance is rather connected with the cell division cycle.

In Fig. 3 besides the changes in alternative respiration, is shown how the ratio of cells being in the "A" phase of the cell cycle (n_A) changes in time; n_A was calculated using the equations

$$\begin{aligned} t < T_B & \quad n_A = e^{-\lambda \cdot t} \\ t \geq T_B & \quad n_A = 2 + (1 - 2 \cdot e^{\lambda \cdot T_B}) \cdot e^{-\lambda \cdot t} \end{aligned}$$

From the fact, that these two parameters change parallel, we may conclude that the development of alternative respiration is connected with the "A" phase of the cell cycle (which is a part of the G_1). The correct answer to the question depends necessarily on examining synchronously proliferating cultures under constant conditions.

The culture studied was obtained by inoculating stationary phase *C. tropicalis* cells into YEPG medium. As the cells of the inoculum were nutrient-starved, they started dividing synchronously in the fresh medium, where during the first cell division cycle the changes in nutrient concentrations were not

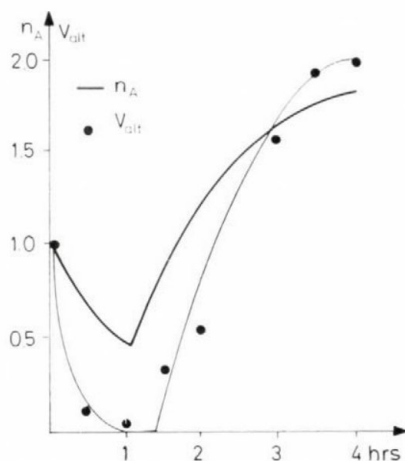


Fig. 3. Changes in the activity of alternative oxidases (—) and the ratio of cells (●—●) being in the "A" phase of the cell cycle

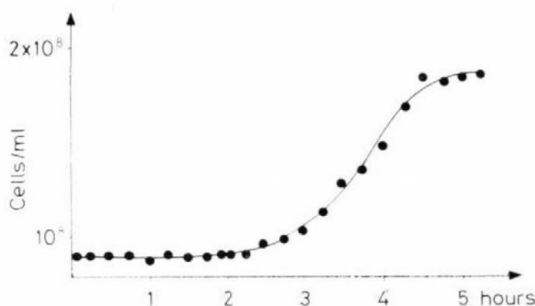


Fig. 4. Change in cell density during synchronous proliferation of *C. tropicalis*

significant. Figures 4 and 5 show the cell density and cell size distribution changes in time. The size distribution at zero time indicates that the inoculum was a mixture of two subpopulations having different average sizes. The peak at $15 \mu\text{m}^3$ comes from the small daughter cells separated from the mother cells during the starvation (first subpopulation), while the other at $20 \mu\text{m}^3$ is built up from the mother cells bigger in size.

During the first two hours of cultivation, the cell size distribution moves to the right, while cell density remains the same, indicating that the size of both mother and daughter cells increases. After the second hour, the cell-density increases and the "shoulder" of the curve decreases which is due to the division of the second subpopulation. At 3.25 h the division rate accelerates as a result of the division of the first subpopulation. At 4.5 h the cell density reaches the double of its starting value, since by this time all the cells have divided once. After this cell density remains constant for a short period, but the size of the

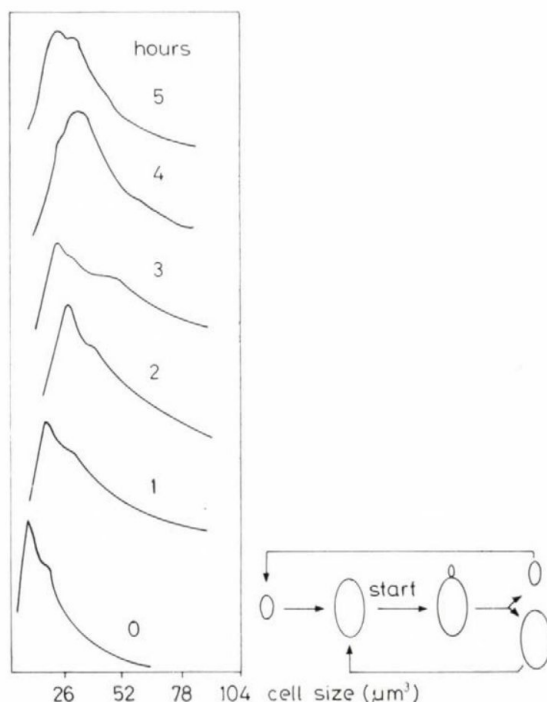


Fig. 5. Change in cell size distribution during synchronous proliferation

cells still fail to show a sole average value, because cells that divided soon after the second hour are larger.

In Fig. 6 the cyanide-resistant respiration changes periodically, having three maximums. The fact of periodicity evidently proves that glucose cannot be responsible for the changes in cyanide-resistance. If alternative respiration were repressed by the glucose, the cyanide-resistance would have to decrease monotonously, which is not supported by the result. Therefore, the periodic changes in the cyanide-resistant respiration might be explained by the cell size distribution. The mother cells in the inoculum (second subpopulation) are in the "A" phase of the cell cycle thus their respiration is cyanide insensitive. The small daughter cells (first subpopulation) are also in the "A" phase but they do not have alternative oxidases, since they are stopped at an earlier event of the cell cycle compared to the mother cells.

Accordingly, the cyanide-resistant respiration observed at the beginning of our experiment is the result of the alternative respiration of the second subpopulation, and it decreases in time as these cells gradually leave the "A" phase. The explanation for the increase and thereafter the decrease of cyanide-resistant respiration is that the cells of the first subpopulation reach more or

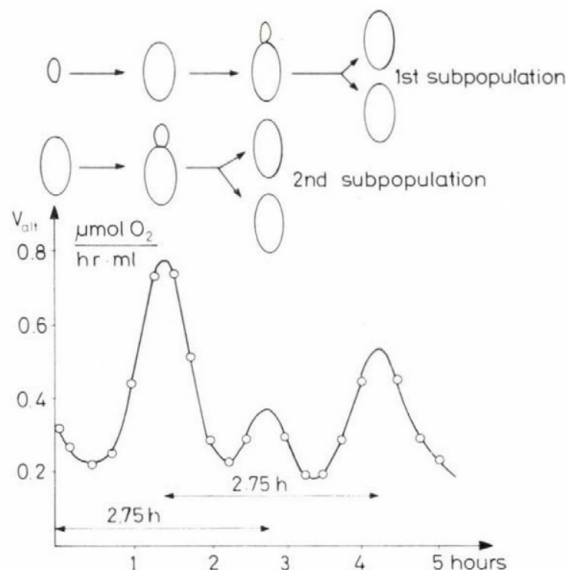


Fig. 6. Time course of the change in cyanide-resistant respiration

less synchronously that point of the cell cycle where respiration is cyanide-insensitive and afterwards, as they carry on the cell cycle, the alternative respiration starts to decrease.

The maximum observed before the third hour is due to the entering the "A" phase of the second subpopulation after the division. The increase of the alternative respiration at 4.25 h is the result of the first subpopulation entering the "A" phase after the division. The above hypothesis is supported by the fact that the time passed between the first and third maximum is exactly as long as from the beginning of the experiment until the second maximum.

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RING-FORMING, OLIGOTROPHIC *MICROCYCLUS* ORGANISMS IN THE WATER AND MUD OF LAKE BALATON

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Seventeen oligotrophic *Microcycilus* strains were isolated from water- and mud-samples obtained in the region of the Keszthely-Bay (Lake Balaton, Hungary) using different synthetic media with 1% $\text{Na}_2\text{S}_2\text{O}_3$. Fifteen strains occurred in samples collected in the winter period. For numerical analysis, all isolates were compared with four authentic strains of recognized *Microcycilus* spp. on the basis of 88 selected tests. They proved to be typical members of the species *Microcycilus aquaticus* (Ørskov), considerably differing at the same time from *Microcycilus major*, with the exception of a single, presumably extreme variant.

The *Microcycilus*-type organisms, these “little-known, beautiful”, “fascinating curvy creatures” [1, 2], are widely distributed in aquatic habitats, and in recent years some members of them were subjected to physiological, biochemical and partly also taxonomic studies [2]. Unfortunately, we have little information on their ecology, their role in the metabolism of water microbial communities and types and species existing in nature. One reason for this lack of knowledge may be their considerable morphological similarity (at least under certain growth conditions) to other also ring-like cell-forming, but not *Microcycilus*-type bacteria as e. g. *Chlorobium*, *Cytophaga*, *Myconostoc*, *Rhodocyclus*, etc., which made them hardly recognizable in extensive, quantitative water bacteriological studies. Consequently, their isolation in pure cultures and correct taxonomic identification are prerequisites of all approaches to their ecology.

The aim of our work was to study and identify the representatives of the *Microcycilus* population in the Lake Balaton, about which we have had so far no information at all, as well as to contribute to our knowledge on the ecology of these less-known organisms.

Materials and methods

Sampling sites. Water- and mud-samples were collected aseptically, in January and April, 1983 in the area of the Keszthely-Bay.

Isolation of strains. Synthetic agar media recommended by Parker and Prisk [3] and by Schneider et al. [4], modified by us and containing 1% $\text{Na}_2\text{S}_2\text{O}_3$ were used for plating. Incubation lasted for 14–30 days at 28 °C.

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Maintenance of strains was performed by transfers on the isolation media and also on nutrient slants yielding a more intense growth, i. e., our strains were not obligate oligotrophs.

Reference strains. Four authentic strains, obtained from international culture collections, were used: *M. aquaticus* CCM-1786, CCM-2548, CCM-2549; *M. major* BKM-B-859.

Cultural and morphological characters. Colour, elevation, margine and viscosity of the colonies were observed on synthetic and complex media. Shape, size and arrangement of cells were studied in Gram-stained smears.

Physiological and biochemical tests. Motility was studied in semisolid Bacto SIM Medium (Difco) after 24 h incubation at 28 °C. NaCl tolerance and growth at different pH values were detected in nutrient broth after 7 and 14 days incubation. Bacto Anaerobic Agar was used to test the ability of growth under anaerobic conditions. Catalase reaction: some drops of a 10% H_2O_2 solution were added to a nutrient slant culture and the development of bubbles was observed. For the Voges-Proskauer reaction and methyl-red test, glucose-phosphate broth was used. Acetoin was detected according to Barritt [5]. Oxidase reaction was tested with 24 h cultures using Pathotec Co. Cytochrome Oxidase paper strips. Nitrate reduction was tested by the method of Gordon et al. [6]. Utilization of salts of organic acids as sole sources of carbon: the basal medium employed by Gordon [7] was adopted. Utilization of citrate as sole C-source was tested on Simmons' citrate-agar medium read after 7 and 14 days at 28 °C. The basal medium described by Gordon and Mihm [8] was employed to study the utilization of 19 different carbohydrates as sole C-sources. The utilization of different N-compounds as N-sources was tested by the method of Tsukamura [9]. Phenylalanine deamination was studied according to Ewing et al. [10]. Indole production was detected with Kovács's reagent [11]. The methods of Cowan and Steel [12] were employed for studying phosphatase activity and the hydrolysis of starch and aesculin. The breakdown of arginine after 6 days incubation was detected in arginine-glucose broth using Nessler reagent. The production of H_2S was studied in Bacto SIM Medium (Difco). The method of Christensen [13] was employed to show urease activity. Gelatinase activity was tested with the aid of Charcoal discs (Oxoid). Difco Agar Base amended with sterile bovine blood was used to detect haemolytic activity. The sensibility to antimicrobial substances was tested by using antibiotic sensibility discs (Institute for Serobacteriological Production and Research "Human", Budapest).

Numerical analysis. Our 17 *Microcycylus* strains were numerically compared to each other and 4 authentic *Microcycylus* spp. strains on the basis of 88 selected and coded diagnostic features. At first the similarity coefficient (S) offered by Sokal and Michener [14] was calculated for every pair of strains by using a Control Data B-300 type computer. Thereafter, cluster-analyses were carried out and dendrograms were prepared employing programs elaborated by Dr. Pál Tőke (L. Eötvös University, Budapest).

Results and discussion

Figure 1 shows a dendrogram, based on 88 coded features involving all of our isolates and the authentic strains. Figures 2 and 3 show electron micrographs of cells of *Microcycylus* strains T2-V/2 isolated from the Lake Balaton and *M. aquaticus* CCM-2548. Finally, Table I demonstrates a comparison of our isolates with authentic *Microcycylus* strains involved into the synchronous studies, taking into consideration only some diagnostic key characters.

Figure 1 clearly shows that there is a large cluster comprising 19 strains (16 Balaton-isolates and 3 authentic *M. aquaticus* strains: CCM-2549, CCM-1786 and CCM-2548) which were united at a relatively high (93.5%) similarity level. This cluster can be considered an assemblage of different representatives of a single species the specific epithet of which is designated by the presence of the 3 authentic *M. aquaticus* strains.

Our *M. aquaticus* strains, representing a local population of this species, are characterized in general by very small Gram-negative, non motile, curved

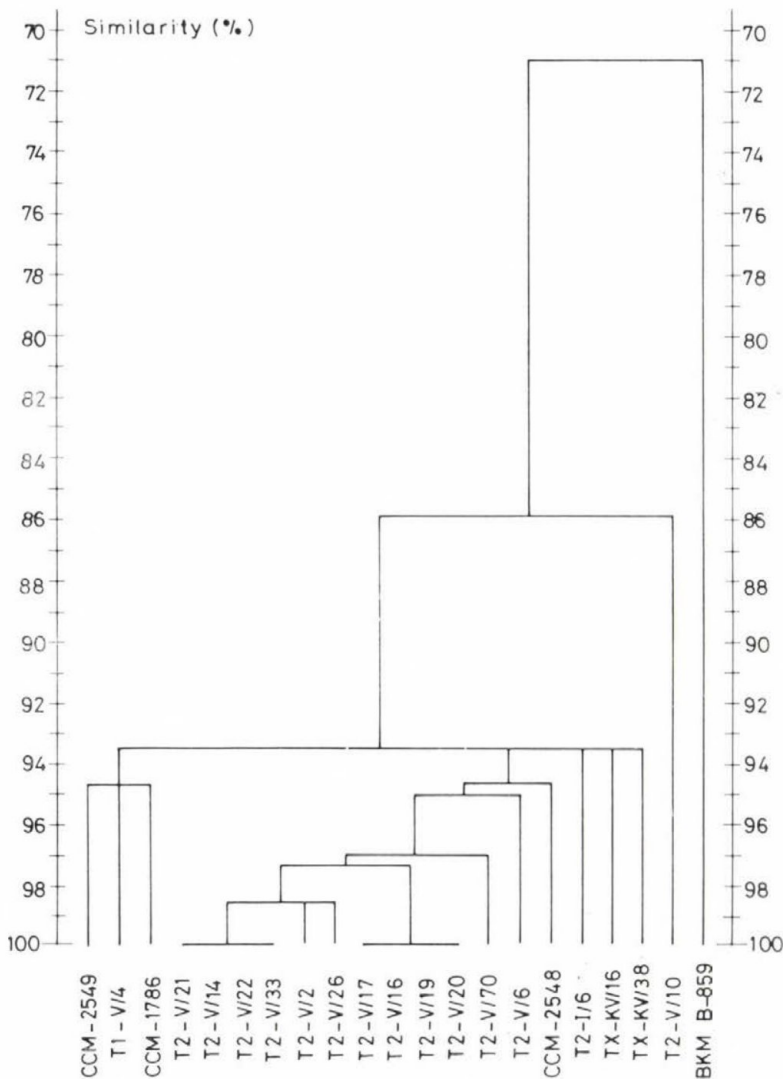


Fig. 1. Dendrogram showing the similarity and phenetic relatedness of 17 *Microcycylus* strains (T1-V/4; T2-V/21; T2-V/14; T2-V/22; T2-V/33; T2-V/2; T2-V/26; T2-V/17; T2-V/16; T2-V/19; T2-V/20; T2-V/70; T2-V/6; T2-V/6; TX-KV/16; TX-KV/38; T2-V/10) isolated from mud and water samples of Lake Balaton and 4 authentic strains of *Microcycylus* spp. (see Table I)

rods (0.4–0.5 μm wide and 1.2–1.5 μm long) with rounded ends. They are oxidase-, catalase- and urease-positive, obligately aerobic organisms, which do not produce indole. Of the 19 carbohydrates tested they utilized only ribose, L-arabinose, adonitol, D-fructose, D-glucose and mannitol. Of the organic acids studied, acetate, lactate and succinate were used by them for growth, but

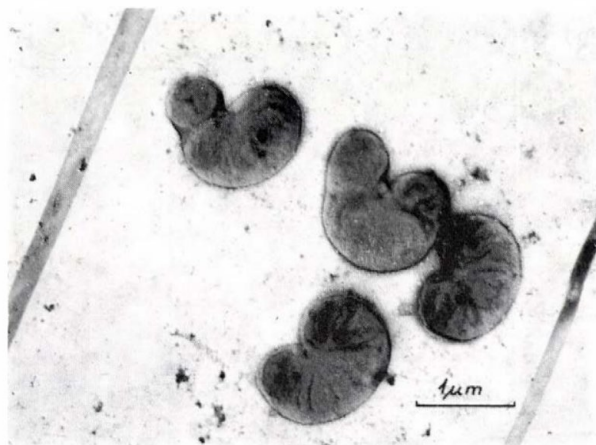


Fig. 2. Electron micrograph of cells of *M. aquaticus* strain T2-V/2 isolated from the Lake Balaton (Photo E. Contreras)

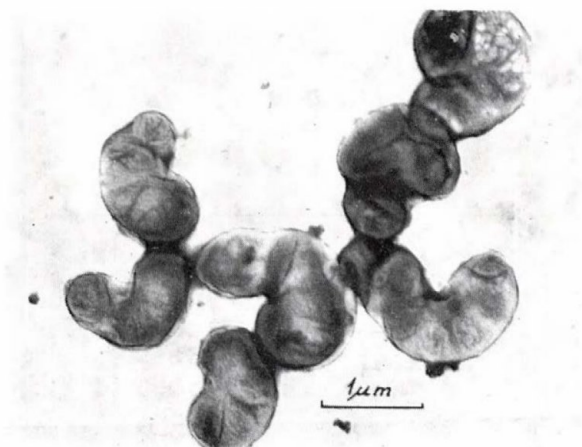


Fig. 3. Electron micrograph of cells of *M. aquaticus* strain CCM-2548 (Photo E. Contreras)

benzoate, gluconate, malate and tartarate not at all. Asparagine proved to be an excellent N-source for them.

Some differences were, however, found between the Balaton and the authentic *M. aquaticus* strains. E. g. the majority of the former could slowly decompose starch, while the latter failed to do so. In contrast, all authentic strains grew on Simons' citrate-agar, but in this respect, only three Balaton-isolates were positive, etc.

As apparent from the dendrogram (Fig. 1), our strain No. T2-V/10, showed only a low level phenotypic similarity to this computer-created *M. aquaticus* cluster with which it was united at 85.8% level. Since its diagnostic key charac-

ters proved to be identical with those of *M. aquaticus*, it may be assumed that it represents an extreme variant of this species differing from the typical members in several physiological properties. E. g. strain T2-V/10 has a relatively narrow C-source utilization spectrum and produces acids only from glucose. It does not utilize organic acids as sole sources of carbon, and cannot grow with asparagine or tryptophan as single N-source etc.

The presented dendrogram (Fig. 1) also shows that strain BKM-B-859 of *M. major* was considerably separated from all other *Microcycilus* strains with which it was united into a single large cluster only at 70.9% similarity level. This finding is in accordance with literary data. On the basis of comparative measurements on guanine+cytosine ratios in the DNAs of *Microcycilus* spp. [2, 15, 16], it seems obvious that *M. aquaticus* and *M. major* are only loosely

Table I

A comparison of selected diagnostic properties of 17 *Microcycilus* strains isolated from water- and mud-samples of Lake Balaton with those of authentic strains of *Microcycilus* spp.

Diagnostic properties	17 <i>M. aquaticus</i> strains isolated from the Lake Balaton	<i>M. aquaticus</i> CCM-2548	<i>M. aquaticus</i> CCM-2549	<i>M. aquaticus</i> CCM-1786	<i>M. major</i> BKM-B-859
Cell diameter, μm	0.4–0.5 (17)	0.4–0.7	0.4–0.7	0.4–0.7	0.7
Length of cells, μm	1–2.5 (17)	1–2.5	1–2.5	1–2.5	5
Colour of colonies	grayish white (17)	grayish white	grayish white	grayish white	rose
Starch hydrolysis	+(11)	—	—	—	+
Citrate utilization Simmons'	+(3)	+	+	+	—
Nitrate reduction	—(17)	—(?)	+	+	—
Phosphatase activity	—(17)	—	—	—	+
C-source utilization					
Adonitol	+(16)	+	+	+	—
Rhamnose	—(16)	—	—	—	+
Lactose	—(17)	—	—	—	+
Maltose	—(17)	—	—	—	+
Raffinose	—(17)	—	—	—	+
Dextrin	—(17)	—	—	—	+
Inulin	—(17)	—	—	—	+
Salicin	—(17)	—	—	—	+
α -methyl-D-glucoside	—(17)	—	—	—	+
Mannitol	+(16)	+	+	+	—
D-Sorbitol	+(15)	+	+	+	—
Utilization of salts of organic acids					
Acetate	+(16)	+	+	+	—
Gluconate	—(16)	—	+	+	+
Lactate	+(16)	+	+	+	—
Sensitivity to gentamicin (20 μg); radius of inhibition zone, mm	4.5–11.5	10.5	7.5	10.5	0

related organisms. At present some workers suggest the separation of these two species at generic level [17, 18]. In the latest edition of Bergey's Manual [19] Larkin and Borrall placed *M. major* into the genus *Flectobacillus* as *F. major* (Gromov 1963) Larkin, Williams and Taylor 1977. According to our studies, in contrast to *M. aquaticus*, strain BKM-B-859 of *M. major* is characterized by relatively larger cells, broader C-source utilization spectrum, rose-coloured colonies, strong amylase and phosphatase activities, resistance to gentamicin etc. (see Table I).

The data in Table I clearly show that the studied bacterial strains characterized by ring-forming cells and isolated from the Lake Balaton are (with the exception of a single strain) typical members of *M. aquaticus*. This species can form relatively large populations both in the mud and open water of the Lake. The majority of our isolates were found in samples obtained during the winter period. This fact correlates with the arousing activities of these organisms in cold water in which otherwise, other oligotrophic elements of microbiota can also increase their population densities. In the future we intend to study the metabolic interrelationships between these interesting bacteria and other members of the complex microbiota of Lake Balaton [20].

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CELL-MEDIATED IMMUNE RESPONSE OF GOATS TO LEPTOSPIROSIS*

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Cell-mediated immunity to *Leptospira pomona* was measured in goats by the leukocyte-migration inhibition test (LMIT) and microassay for stimulation of protein synthesis (SPS) with ^3H -leucine. *L. pomona* soluble antigen was used in both tests. The migration indices in the group of goats infected with *L. pomona* were significantly lower ($< .001$) as compared to control. The migration zones of the unstimulated peripheral blood leukocytes in the infected group were significantly smaller ($< .001$) than those of the control. The SPS test was strongly positive in test animals and was negative in control.

Cell-mediated immunity (CMI) is an important defense mechanism against many microbial infections. Consequently, several *in vitro* methods to detect cellular hypersensitivity have been developed [1–4]. Methods such as the leukocyte migration inhibition test (LMIT) and the microassay for stimulation of protein synthesis (SPS) were explored as a suitable *in vitro* correlate of CMI [5–8]. Although several studies on various infections have been reported, no such work on leptospirosis in goats has been presented. The purpose of the present study was to evaluate the LMIT and SPS tests for leptospirosis in experimentally infected goats.

Materials and methods

Animals. A total of three mixed Nubian goats (1 male and 2 females) were used in the study. Pretrial sera from these goats were titrated and were used as control. These sera were found negative for *Leptospira pomona*. These goats were injected with 5 ml of a 3-day-old culture of *L. pomona*, strain 11000-36A (National Animal Disease Center, Ames, Iowa) containing 1×10^7 organisms per ml by subcutaneous route. The animals were again injected with the same dose and route 3 weeks later. Two weeks following the last injection, the animals were bled and tested for cell-mediated immune response by employing LMIT and microassay for stimulation of protein synthesis.

Assay procedures. The LMIT was carried out as described by other workers [5, 9, 10] with some modifications. Viability of lymphocyte cells as determined by trypan blue dye was found to be 95%. The test was carried out by adjusting the suspensions to contain 12×10^6 to 15×10^6 cells per agarose well. The cells were suspended in TC-199 medium with or without antigen and were placed in wells cut in agarose plates. After incubation for 12 h at 37°C in 5% CO_2 ,

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the cells were fixed with 3% glutaraldehyde for 30 min. After removing agarose and repeated washings, the migration was measured. For calculation of percentage migration inhibition, the following formula was used.

$$MI = \frac{x \text{ diameter migration Ag}}{x \text{ diameter migration C}}$$

% Inhibition = $(1.00 - MI) 100$

A MI of ≤ 0.80 and a % inhibition of 20% is considered positive leukocyte migration-inhibition activity.

Preparation of soluble antigen of *L. pomona*. The antigen was prepared as described by Jenkins [3]. The organism was grown in Bovamemar Microbiological Media solution-PLM-5 (Amour Pharmaceutical Laboratory, Kankakee, Illinois). The organisms were centrifuged, washed and subjected to sonification. The disruption of cells was monitored microscopically. The supernatant was treated with trichloroacetic acid (TCA: 10% by dry weight) and recentrifuged. The precipitate so obtained was dialyzed for removal of TCA. After adjustment of pH to 7.0, the suspension was passed through a $0.22 \mu\text{m}$ (APD) filter (Millipore Corporation, Bedford, MA), and the protein content was determined by the method of Lowry et al. [11]. The antigen obtained was then stored at -20°C until used later.

Stimulation of protein synthesis. Fifteen ml of heparinized blood were layered on 4.5 ml of Ficoll Hypaque and centrifuged at 2000 rpm for 30 min at room temperature. Recovered lymphocytes were counted and adjusted to contain 1×10^6 to 2×10^6 cells/ml in minimum essential medium (MEM) without leucine but containing L-glutamine $10 \mu\text{g/ml}$, penicillin 100 U/ml , streptomycin $100 \mu\text{g/ml}$, essential vitamins $10 \mu\text{g/ml}$ and buffered to $\text{pH } 7.2 \pm 0.2$.

The antigen was diluted to obtain the desired concentration of 25, 50 and $100 \mu\text{g}$ protein/ml. The protein content of the antigen was determined by the method of Lowry et al. [11]. The phytohemagglutinin (PHA) was also diluted to 1 : 10 and 1 : 100. All dilutions were made in MEM and 0.02 ml of the antigen was added to each well in microtitration plate and in quadruplicates. Samples (0.2 ml of 1×10^6 cells/ml) of lymphocytes from sensitized and unsensitized animals were suspended in MEM supplemented with 50 units of penicillin/ml and $50 \mu\text{g/ml}$ of streptomycin. A similar suspension of lymphocytes was added to phytohemagglutinin (PHA) or in MEM as dictated by the experimental protocol.

The lymphocyte culture was incubated 18–24 h at 37°C in a humidified atmosphere of 5% CO_2 . Following incubation, 0.05 ml of MEM containing 1 Ci and ^3H -leucine (sp. act. 40–60 Ci/m mol) was added to each well the lymphocyte cultures were incubated for an additional 6 h and then washed with 2% acetic acid on glass fiber filters, using a multiple sample harvester. The filters were desiccated and placed in 4.5 ml of scintillation fluid, and the radioactivity was measured in a beta liquid scintillation counter. Results were expressed as counts per min (cpm) or as stimulation index (SI) which was determined by dividing the mean cpm of unstimulated cultures [7, 8].

$$SI = \frac{\text{cpm in stimulated cultures}}{\text{cpm in control cultures}}$$

Results

Leukocyte migration-inhibition test. An evaluation of the response to leptospira in goats was demonstrated by use of LMIT (Table I). All the animals were tested for reactivity to leptospiral antigen at different concentrations. A migration index (MI) of ≤ 0.80 indicated a positive reaction for LMIT. All 3 goats had positive migration inhibition indices to the immunizing antigens at a concentration of $50 \mu\text{g/ml}$ protein. Goat No. 11 showed positive reaction at a concentration of 50 and $25 \mu\text{g/ml}$ protein of *L. pomona* antigen while goats Nos 12 and 14 had positive inhibition to 100 and $50 \mu\text{g/ml}$ protein.

Lymphocyte stimulation assay. The results of lymphocyte stimulation assay (LSA) with different concentration of *L. pomona* antigens and different dilutions

Table I

*Leukocyte migration-inhibition test of sera from animals infected with *L. pomona**

Designation of goat	Immunizing antigen	Mean migration index antigen concentration in $\mu\text{g/ml}$		
		100	50	25
11	<i>L. pomona</i>	0.85	0.69*	0.64*
12	<i>L. pomona</i>	0.69*	0.76*	1.00
14	<i>L. pomona</i>	0.77*	0.77*	1.00

* $\text{MI} \leq 0.80$ = positive for inhibition

Table II

*Stimulation of protein synthesis with different concentrations of *L. pomona* antigen and with various dilutions of phytohemagglutinin-M*

Antigen concentration $\mu\text{g/well}$	Designation of goat		
	11	12	14
—	3.72 ± 1.75	1.510 ± 1.12	1.030 ± 1.23
25	8.363 ± 2.07	5.010 ± 1.89	11.867 ± 3.14
50	22.545 ± 6.47	20.787 ± 5.10	16.821 ± 6.04
100	15.643 ± 5.67	13.289 ± 6.43	11.683 ± 5.63
Mitogen (PHA-M) dilution/well			
1 : 10	6.163 ± 3.63	4.087 ± 2.89	7.364 ± 3.62
1 : 100	14.169 ± 4.89	11.369 ± 4.01	10.386 ± 5.69

Counts per minute $\pm$ standard error (1×10^3)

of phytohemagglutinin (PHA-M) are shown in Table II. The result indicated that the antigen concentrations of 25, 50, 100 g protein per well and the two PHA (mitogen dilutions) 1 : 10 and 1 : 100 dilution per well were greater in their influence on cpm for the LSA of the infected animal with *L. pomona* to that of the control. The highest cpm were observed on an antigen concentration of 50 μg protein per well for all the animals. Mitogens at 1 : 100 dilution gave the highest cpm when compared with control.

Discussion

As appears from the results, the migration zones of the antigen-free cell suspensions from the goats infected with *L. pomona* are larger than those treated with antigen. The diminished migration could be due to the cells being

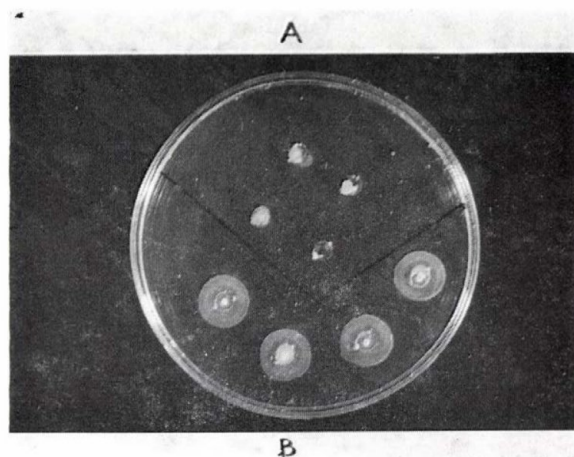


Fig. 1. A leukocyte migration inhibition test (Goat No. 14). (A) Leukocytes from infected goat in the presence of soluble antigen of *L. pomona*; (B) leukocytes in the absence of antigen. Measurements were made of diameter of migration zone

affected by in vitro produced leukocyte-migratory inhibitory factors [10, 12-15]. The total number of cells per well was not a critical factor as well as the period of preincubation. However, the optimum number of cells per well was found to be in the range of 12×10^6 to 15×10^6 cells per well. The maintenance of proper pH in the cell culture and agarose medium was very critical for demonstration in inhibition because the activity of the inhibitory factor was found to exist within a narrow range of pH 7.2 to 7.4.

The microprotein assay for lymphocyte stimulation with leptospiral antigen was used to detect CMI reactivity in goats infected with *L. pomona*. Similar responses have been reported by other workers relating to various infectious agents [1-4, 6-8, 12-15]. After exposure to leptospirae, detectable sensitization of peripheral blood lymphocytes was induced in infected animals which indicated the presence of sufficient sensitized cells to evoke a CMI response. Further, the results obtained indicate that leptospirae have the ability to stimulate cell-mediated immunity in the host.

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SUBDIVISION OF COMMON *SALMONELLA* SEROTYPES: PHAGE TYPING OF *S. VIRCHOW*, *S. MANHATTAN*, *S. THOMPSON*, *S. ORANIENBURG* AND *S. BAREILLY*

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The phage typing system elaborated for *Salmonella infantis* proved suitable to sub-classify 633 *S. virchow*, 206 *S. manhattan*, 103 *S. thompson*, 30 *S. oranienburg* and 58 *S. bareilly* strains. The *S. virchow* strains belonged to phage type 213 in 79.6%, each of six phage types occurred more frequently than 1%, and 15 phage types less frequently than 1%. The *S. manhattan* strains belonged to three phage types (687, 247, 547) in 91.2% and nine other types were found. The most common phage types among the *S. thompson* strains were 243 and 247 (52.4 and 18.4%) and eleven other types were encountered. All *S. oranienburg* strains derived from an outbreak and belonged uniformly to phage type 111. *S. bareilly* strains isolated from an other outbreak fell into phage type 683.

For an effective epidemiological tracing of common *Salmonella* serotypes, determination of the antigenic structure should be supplemented with methods allowing a further subdivision. For this purpose, by the use of the *S. infantis* phage set, attempts were made to subdivide *Salmonella* serotypes belonging to group C.

Materials and methods

Bacterial strains. Phage type was determined of 633 *S. virchow*, 206 *S. manhattan*, 103 *S. thompson*, 30 *S. oranienburg* and 58 *S. bareilly* strains isolated in 1985–1986 by the bacteriology laboratories of Hungarian Public Health Stations.

Determination of phage type. The second version of the method elaborated for phage typing of *S. infantis* was used [1].

Media. Oxoid nutrient broth No. 2 and nutrient agar were used.

Results

Phage type distribution of *S. virchow*. A total of 430 *S. virchow* strains of human and 203 strains of animal and food origin were isolated between 1985 and 1986 (Table I). The majority of the human and animal strains belonged to phage type 213 (83.3% and 71.9%, respectively). Among the human strains phage types 113 and 143 were more frequent than 5% in 1985 and phage type

243 in 1986; among the non-human strains phage types 243, 248 and 211 occurred more frequently than 5%. Eight phage types were more frequent than 0.5%, 13 types (215, 217, 245, 247, 413, 517, 535, 615, 623, 633, 687, 688, 837) occurred less than in 0.5%. Out of the rare phage types 217, 245, 517, 613, 633 and 688 were found only among the non-human strains. The animal strains were isolated from chicken, turkey, goose and dog; the strains of food origin were derived from egg, eggpowder, noodles and pork. Phage type 213 was ubiquitous, type 113 was found in counties Csongrád and Békés, type 143 in Borsod, type 243 in Heves and Pest. Phage type 213, too, was the most frequent among the strains of animal and food origin, but types 243 and 248 were also frequent in counties Baranya and Békés.

Table I

Percentage distribution of *S. virchow* phage types in the years 1985 and 1986

Phage type	Origin of strains				Total
	human		animal, food, hygienic control examination		
	1985	1986	1985	1986	
113	6.8	0.7	—	—	3.2
143	5.0	—	—	—	2.2
211	—	—	—	7.5	1.1
213	81.3	86.7	60.9	84.9	79.6
243	2.9	8.6	14.5	1.1	6.0
248	—	—	14.5	—	2.5
513	2.5	0.7	1.8	1.1	1.7
543	0.4	—	0.9	1.1	0.5
613	—	—	1.8	3.2	0.8
Other					
13 kinds	1.1	3.3	5.6	1.1	2.4
No. of strains	279	151	110	93	633

The first outbreak caused by *S. virchow* in Hungary took place in a college for girls in Székesfehérvár (Fejér county) in May, 1985. Out of the 500 persons consuming the food prepared in the college kitchen, 187 became ill. The infection was conveyed by stuffed chicken. At the time of the investigation food samples were not available, but from the raw chicken-meat as well as from the stool of 55 patients and 59 asymptomatic excretors *S. virchow* was isolated and all strains belonged uniformly to phage type 213.

Almost simultaneously with the above-mentioned outbreak, the first hospital outbreak associated with *S. virchow* in Hungary, occurred in Eger. In the infection all wards of the Heves County Hospital were involved and 271 cases were recorded. *S. virchow* strains of phage type 213 were isolated from the stools of patients, asymptomatic excretors and from the pork prepared in the hospital's kitchen.

A community outbreak was caused by *S. virchow* in a village (Miske, Békés county) in May, 1986. Out of the 400 participants of a wedding-party 66 persons got ill. *S. virchow* was isolated from the stools of 27 patients, 16 asymptomatic excretors and from six different food samples of the wedding dishes. The strains isolated from the patients and food samples belonged to phage type 213.

Phage type distribution of S. manhattan. The 206 strains — 185 of human, 13 of animal and 8 of water origin — fell into 12 phage types (Table II). The strains belonged to three frequent phage types (687, 247, 547) in 91.2%. Three

Table II

Phage type distribution of S. manhattan strains in the years 1985 and 1986

Phage type	No. of strains		Total	
	1985	1986	No.	%
243	—	1	1	0.5
247	11	48	59	28.6
267	1	—	1	0.5
547	13	40	53	25.7
647	—	3	3	1.5
667	—	1	1	0.5
677	—	2	2	1.0
687	—	76	76	36.9
847	—	4	4	1.9
867	—	1	1	0.5
885	—	1	1	0.5
887	—	4	4	1.9
Total	25	181	206	100.0

phage types were more frequent than 1% and the frequency of six phage types was less than 1%. In Heves county the most frequent was phage type 687, followed by types 247 and 547. In Somogy type 547, occurred most frequently, in Veszprém, phage type 247 was first, and type 547 next in order.

Strains of phage type 547 were isolated from patients and food samples in course of a community outbreak that took place in a girls' college in Budapest. Another outbreak, too, was caused by this type in an old people's home. An outbreak was associated with phage type 687 in a rest-house; the agent was isolated from the patients and from the food, responsible for the spread of infection.

The epidemiological role of *S. manhattan* during these years was significant in certain areas (counties Veszprém and Tolna and Budapest) the infections being spread mainly by meat products.

Phage type distribution of S. thompson. Out of the examined 103 strains 95 were of human, 4 of animal or food and 4 of water origin. Table III shows the

Table III

Phage type distribution of S. thompson strains in the years 1985 and 1986

Phage type	No. of strains		Total	
	1985	1986	No.	%
141	1	—	1	1.0
143	—	4	4	3.9
213	—	6	6	5.8
243	33	21	54	52.4
247	19	—	19	18.4
283	1	—	1	1.0
513	1	—	1	1.0
543	—	1	1	1.0
547	1	—	1	1.0
643	—	1	1	1.0
647	—	2	2	1.9
683	6	3	9	8.7
687	—	3	3	2.9
Total	62	41	103	100.0

distribution of phage types. The strains belonged to 13 phage types. The most frequent was phage type 243, including more than half of the strains (52.4%). Phage type 247 was also frequent (18.4%). The incidence of five phage types varied between 1 to 10%, six phage types occurred only in 1%. Strains of phage type 683 derived from community outbreaks, whereas in family infections phage types 243 and 247 occurred.

Phage sensitivity of S. oranienburg. In an outbreak in Győr-Sopron county, 30 strains isolated from patients, excretors and from the incriminated sausage, belonged uniformly to phage type 111.

Phage type of S. bareilly. During an outbreak in Gyula (Békés county), lasting for several months in wintertime in 1985–1986, 279 patients were reported. The pathogenic agent spread via meat-products of different abattoirs and by poultry, the latter being indirectly verified by hygienic-bacteriological and epidemiological examinations. A connection between the cases, occurring in different localities and at different times, was indicated by the uniform phage type (683) of the strains, isolated from the infected persons, incriminated foods and at hygienic control examinations.

Discussion

There are phage typing methods to subdivide the most important *Salmonella* serotypes. Phage typing of *S. typhi* was elaborated first and it has been used world-wide since the forties [2, 3]. Methods were published for the phage typing of *S. paratyphi*-B [4–6], *S. typhi-murium* [4, 7, 8], *S. panama* [9], *S.*

enteritidis [10, 11] and *S. infantis* [12, 1]. The possibilities to subdivide other common *Salmonella* serotypes are rather limited. The phage typing methods developed for *Salmonella* serotypes were summarized by Guinée and van Leeuwen [13]. Methods published for *S. adelaide* [14], *S. anatum* [15], *S. bareilly* [16, 17], *S. blockley* [18], *S. bovis-morbificans* [19], *S. brandenburg* [20], *S. binza* [21], *S. dublin* [22, 23], *S. gallinarum-pullorum* [24, 22], *S. good* [25], *S. heidelberg* [26], *S. newport* [27, 28], *S. senftenberg* [29], *S. cholera-suis* and *S. typhi-suis* [30] and *S. weltevreden* [31–33], have not been used widely.

A method was elaborated by Velaudapillai [34] to subdivide *S. virchow*. The examined 55 strains were classified into seven phage types using five phages obtained from lysogenic strains.

S. thompson was subdivided by Smith [35] by the use of six temperate phages possessing different host ranges. Out of the examined 50 strains 41 fell into 11 phage types, 9 strains were not typable. The epidemiological application of the method was not reported. Anderson [36] also elaborated a method for *S. thompson* but did not publish details. Gershman [37] subdivided *S. thompson* into 13 phage types using phages isolated from sewage.

In Hungary, the number of human salmonellosis is gradually increasing year by year, especially in the last two years (1985, 1986), when the number of isolations (positive persons) amounted to 13 617 and 17 488, respectively. The incidence of *S. virchow* and *S. manhattan* isolations became significant besides the three most common serotypes (*S. enteritidis*, *S. typhi-murium*, *S. infantis*) in 1985–1986.

The unexpected rapid spread of *S. virchow* infections and the continuous increase in the number of *S. manhattan* isolations demanded a detailed analysis of the epidemiological characters of these serotypes.

The regular occurrence of *S. virchow* has been observed in Hungary since 1982; in 1984 it fell into the 27th rank only, but spreading all over the country it became the fourth in 1985. The ratio of the strains isolated from epidemic outbreaks (47.9%) and from sporadic cases (52.1%) was nearly the same in 1985, the number of patients was one and a half times higher than that of the asymptomatic excreters. The number of isolations decreased slightly in 1986 and fell into the 5th rank in the order of frequency. Simultaneously, the ratio of the epidemic occurrence decreased significantly (28.8%), the majority of the isolates (71.2%) originated from sporadic cases. The infections with clinical symptoms occurred much more frequently than previously, the ratio of patients being two and a half times higher than of excreters.

The frequency of *S. manhattan* isolations increased continuously in the last years; the serotype occupied the 6th rank in 1985, and the 4th in 1986. There was no significant change in the patient/excreter ratio in the last two years: the number of persons with clinical symptoms was nearly two and a half times higher than the number of excreters. In 1985, nearly half of the isolations

were derived from epidemic outbreaks and from sporadic cases; in 1986 the ratio of sporadic cases increased significantly, becoming nearly two and a half times higher than the epidemic cases.

The majority of the *S. thompson*, *S. oranienburg* and *S. bareilly* isolates were derived mainly from persons with clinical symptoms of salmonellosis. Phage typing of the strains allowed to prove the common source of infections in certain epidemics, and confirmed the conclusions made on the basis of epidemiological investigations.

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INTERFERON PRODUCTION IN MYELO- AND LYMPHOPROLIFERATIVE DISEASES

I. SPONTANEOUS INTERFERON PRODUCTION IN ACUTE AND CHRONIC LEUKAEMIAS

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Blood plasma interferon (IFN) of patients with acute myeloid (AML) and chronic granulocytic (CGL) and with acute and chronic lymphoid leukaemia (ALL and CLL) was measured by bioassay and characterized by neutralization with anti-human-IFN- α treatment. Elevated IFN- α level (60–125 IU/ml) was found in the quiescent phase of CGL as compared to the control samples (15 ± 10 IU/ml). No significant differences could be found between plasma IFN of patients suffering from the blastic crisis of CGL and control persons. Analogous results were obtained in experiments with plasmas of AML patients, regardless the stage of the disease. Elevated IFN- α production (60–100 IU/ml) was found in patients being in the remission phase of T-cell ALL but only in one case in the progressive phase of the disease. No significant elevation of plasma IFN level was demonstrated in patients with O-cell ALL and B-cell CLL, as compared to the control samples.

Interferons (IFNs) play a role in defense mechanisms against different infections, particularly in the course of viral diseases. IFNs are also endowed with antitumour activities not only in animal systems but in some cases in humans, too (for a review see [1]). It is well documented that IFN is successfully used in the treatment of leukaemias. The most impressive responses have occurred in hairy cell [2] and chronic granulocytic [3] leukaemia. Moreover, to our knowledge no data are available concerning the spontaneous IFN production in different types of myelo- and lymphoproliferative diseases. In the present paper we report investigations on the activation of IFN system in patients with myeloid and lymphoid leukaemias. The possible role of activated IFN system in leukaemic patients is discussed.

Materials and methods

Blood plasma samples. The blood samples of patients with AML, CGL, ALL and CLL as well as of 20 healthy control persons were obtained by venipuncture in the morning. Plasmas were stored at -70°C until used, usually within 4 weeks. All subjects denied the existence of viral illnesses, furthermore, the physical examination did not reveal signs and symptoms of viral infection.

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Cell cultures. The interferon-sensitive WISH human amnion cells and Madin-Darby bovine kidney (MDBK) cells were kindly provided by Professor Ilona Béládi (Institute of Microbiology, University Medical School, Szeged, Hungary). Both cell cultures were grown in Dulbecco's modified Eagle's medium in the presence of 5% fetal calf serum (Gibco Bio-Cult Ltd., Paisley, Scotland).

Interferon (IFN) assays. The plasma samples were tested for their IFN activity in cytopathogenic effect inhibition assays as previously described [4]. In brief, 2×10^4 WISH cells were incubated with twofold serial dilutions of plasma samples in 96-well microtiter plates and then challenged with vesicular stomatitis virus. Virus-induced cytopathology was evaluated microscopically at 24–30 h after infection. The assays included a reference IFN α which was standardized to NIH-G-023-901-527. The titres of triplicated plasma samples are expressed in International Units (IU) per ml.

For characterization of IFN, assays were performed in duplicate on WISH and MDBK cells. Further characterization of IFN was done by neutralization using anti-human IFN- α (anti-Hu-IFN- α) antiserum (a generous gift from Dr. I. Mécs, Institute of Microbiology, University Medical School, Szeged, Hungary). The polyclonal, goat IFN- α -specific antiserum was diluted to neutralize 500 IU/ml of IFN- α . After an incubation at 37 °C for 1 h the samples were assayed for the IFN activity.

Results

Plasma samples obtained from the quiescent phase and the blastic crisis of 10 patients with CGL were assayed for IFN content. Results are summarized in Table I. Elevated IFN levels were found in the quiescent phase in 9 of 10 persons (60–125 IU/ml) as compared to the values of control samples (15 ± 10 IU/ml). On the basis of parallel titration on WISH and MDBK cells as well as neutralization with anti-Hu-IFN- α immune serum, this IFN activity proved to be IFN- α . On the contrary, we failed to detect significantly elevated IFN levels in the blastic crisis of the same patients.

Table I

Detection and characterization of IFN in plasma samples of patients with different phases of CGL

Patient No.	Phase of disease							
	quiescent				blastic			
	IFN activity on cells (IU/ml)							
	WISH		MDBK		WISH		MDBK	
	before	after	before	after	before	after	before	after
	anti-Hu-IFN- α treatment				anti-Hu-IFN- α treatment			
1	60	—	60	—	30	20	20	—
2	40	—	20	—	—	—	—	—
3	125	20	60	—	10	10	—	—
4	80	—	80	—	—	—	—	—
5	70	25	60	—	—	—	—	—
6	100	—	100	—	20	15	—	—
7	95	—	70	—	—	—	—	—
8	80	20	60	—	—	—	—	—
9	75	—	70	—	25	25	—	—
10	60	—	50	—	—	—	—	—

In the case of patients Nos 1, 3 and 6 listed in Table I, we had opportunity to follow the changes in IFN profile during a 30 months period. IFN titres of the three patients in the quiescent, accelerated as well as in blastic phases of CGL are shown in Table II. Results indicate a gradual decrease of the IFN- α level in the course of disease. In the blastic crisis plasma IFN dropped to low or undetectable level.

Table II
Changes in plasma IFN during the course of CGL

Patient No.*	Relative date in months	Phase	IFN activity on cells (IU/ml)			
			WISH		MDBK	
			before	after	before	after
			anti-Hu-IFN- α treatment			
1	0	quiescent	60	—	60	—
	3	quiescent	60	—	45	—
	8	quiescent	40	—	35	—
	15	accelerated	35	10	35	—
	18	accelerated	30	20	20	—
	30	blastic	—	—	—	—
3	0	quiescent	125	20	60	—
	2	quiescent	100	—	65	—
	12	accelerated	60	—	20	—
	18	accelerated	30	20	—	—
	24	blastic	0	0	—	—
	30	blastic	10	10	—	—
6	0	quiescent	100	—	100	—
	3	quiescent	100	20	40	—
	10	quiescent	80	20	—	—
	13	quiescent	45	30	—	—
	24	accelerated	25	25	—	—
	30	blastic	0	0	—	—

Patients listed in Table I

Studies on IFN level of plasma samples from 9 patients with acute myeloid leukaemia gave negative results regardless the stage of the disease (progression or remission); no significant differences could be found between IFN content of AML and control plasma samples (data are not shown).

Table III summarizes results of investigations on plasma IFN in 10 patients with different types of ALL. Elevated IFN levels were found in 5 patients being in the remission phase of T-cell ALL (60–100 IU/ml). This IFN activity proved to be IFN- γ . On the contrary, progression of the disease was characterized with undetectable IFN-level, except one case (slightly elevated values in patient No. 5). No significant elevation of plasma IFN level could be demonstrated in patients with O-cell ALL, as compared to the control samples.

IFN titrations of plasma samples from 10 patients with CLL resulted in negative (7 cases) or low (15–30 IU/ml) values (data not shown).

Table III

Detection and characterization of IFN in plasma samples of patients with different types of ALL

Patient No.	Cell type	Phase of disease							
		remission				progression			
		IFN activity on cells (IU/ml)							
		WISH		MDBK		WISH		MDBK	
		before	after	before	after	before	after	before	after
		anti-Hu-IFN- α treatment				anti-Hu-IFN- α treatment			
1	T	70	60	—	—	—	—	—	—
2	T	100	100	—	—	—	—	—	—
3	T	70	50	—	—	—	—	—	—
4	T	60	60	—	—	—	—	—	—
5	T	60	50	—	—	45	40	—	—
6	O	30	20	—	—	—	—	—	—
7	O	30	25	—	—	—	—	—	—
8	O	—	—	—	—	—	—	—	—
9	O	—	—	—	—	—	—	—	—
10	O	—	—	—	—	—	—	—	—

Discussion

Elevated IFN- α level was found in the quiescent phase of CGL in the majority of patients. IFNs have well-established antiproliferative activity and also affect cellular differentiation [5]. The beneficial effect of endogenously produced IFNs is probably due to their antiproliferative effect on leukaemic cells together with influencing differentiation during the granulopoiesis. The relatively benign quiescent phase of CGL, characterized by massive expansion of terminally differentiated granulocytes eventually progress to the lethal blastic crisis in which immature blasts predominate [6]. Decrease of IFN level preceding the development of blastic crisis is in concordance with these observations. Therapeutical successes in CGL with IFN- α published by different authors [7,8] suggest that this type of IFN affects the differentiation of leukaemia cells and delays the onset of accelerated phase and/or blastic crisis, presumably by inhibition of oncogene activation [9] and suppression of the Philadelphia chromosome-positive clones [10].

The second open question is the origin of endogenously produced IFN- α . The viral etiology of CGL in animals [11] including primates [12] is widely accepted. Expression of retroviral sequences related to the baboon endogenous virus was detected in the quiescent phase of CGL [13] which may be responsible for induction of IFN- α . This IFN- α might inhibit the activation of oncogene(s) as well as the expression of another endogenous human retrovirus related to

SSV/GaLV which proved to be characteristic for the accelerated phase and blastic crisis of CGL [14]. This assumption must be confirmed by more detailed prospective studies.

Acute myeloid leukaemia resembles blastic crisis of CGL in maturation at level of blasts as well as in clinical picture. Nor surprisingly, no considerable endogenous IFN production can be detected in the blood of patients with AML.

Elevated IFN- γ level was found in 5 patients being in the remission phase of T-cell ALL, but not in patients with the progressive phase of disease, except patient No. 5. On the contrary, no significant elevation of plasma IFN level could be detected in patients with O-cell ALL. The anti-proliferative action [15] and immunomodulation with activation of natural killer cells and macrophages [16] are well established effects of IFN- γ and may be involved in the maintenance of the remission of T-cell ALL. But in the light of the results showing reduced expression of *c-myc* in HL-60 cells induced by IFN- γ [17] the inhibition of activity of oncogene(s) may be another possible mode of its beneficial effect, too. It is known that IFN- γ is produced by lymphocytes on stimulation with T-cell mitogens, specific antigens or T-cell growth factor [18]. Further studies are required to reveal the source of endogenously produced IFN- γ in patients with T-cell ALL. Studies of IFN production of different subpopulations of lymphocytes from patients with T-cell ALL are going on in our laboratory.

On the basis of our results the activation of IFN system presumably has not any role in the pathogenesis of CLL. Similarly, IFN therapy of CLL was ineffective in the majority of clinical trials [19–21].

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ALTERATIONS IN THE CHEMICAL COMPOSITION AND ANTIGENICITY OF *ESCHERICHIA COLI* O89 LIPOPOLYSACCHARIDE AND LIPID A AFTER ^{60}Co GAMMA IRRADIATION

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The chemical composition and the antigenicity of *Escherichia coli* lipopolysaccharide (LPS) and lipid A were investigated after irradiation with 150 kGy ^{60}Co -gamma ray. Compared to the original preparations, the irradiated LPS showed a significant reduction in glucosamine, glucose and galactose and a decrease in the phosphate content. The fatty acid components were reduced to a smaller degree. Irradiation induced a reduction in the antigenicity of the polysaccharide part. The amount of glucosamine and phosphate decreased in the irradiated lipid A. The fatty acid content was significantly reduced. The alteration in the chemical composition was not paralleled by changes in the antigenicity of irradiated lipid A.

It has previously been shown that the biological properties of lipopolysaccharides (endotoxins, LPS) are significantly altered by ^{60}Co gamma irradiation [1, 2]. After the first observations on these effects by Previte et al. [3], not only biological, but also chemical changes of LPS resulting from irradiation have been studied by a number of investigators [4–7]. In the present paper we attempted to identify chemical alterations following the irradiation of LPS. In contrast to a previous study [7] where Re LPS was used, we examined, in addition to lipid A the LPS of a smooth strain, *Escherichia coli* O89, in order to show also changes in the polysaccharide part of LPS. The irradiated LPS (LPS_{irr}) and lipid A (lipid A_{irr}) were analysed comparatively with the untreated preparations. We also tested the antigenic properties of the irradiated products.

Materials and methods

Lipid A and LPS preparations. Lipid A was prepared from the LPS of *E. coli* Re mutant strain 515 [8] and used in the form of its triethyl ammonium salt. The LPS from *E. coli* O89 was isolated by the phenol–water method [9].

Irradiation conditions. LPS (2 samples, each with 10 mg in 1 ml water) and lipid A (8 samples, each with 2.5 mg in 0.5 ml water) were irradiated with a dose of 150 kGy of ^{60}Co gamma. The samples were then freeze-dried [10].

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Chemical analysis. Glucosamine was determined after hydrolysis by the Morgan–Elson method [11], phosphate according to Lowry [12], KDO by the thiobarbituric acid reaction [13]. Fatty acids were transesterified by acetylchloride-methanol and estimated by gas liquid chromatography [14]. Sugars were determined after hydrolysis, reduction and acetylation by gas liquid chromatography [15].

Passive haemolysis inhibition test. Serial dilutions of the inhibitors (parent and irradiated preparations) were incubated with the respective serum (37 °C, 15 min), and the mixture added to antigen-coated sheep red blood cells and complement. After incubation (37 °C, 60 min), the inhibition of haemolysis was determined [16]. The 50% endpoint titers were determined by the naked eye. Antisera against Lipid A, Ra-, Rb-, Rc-, Rd-, and Re-LPS were prepared as described earlier [16]. The *E. coli* O89 antiserum [17] was kindly provided by Dr. Éva Cziráková, (National Institute of Hygiene, Budapest).

Results

Table I shows that the glucosamine and phosphate contents are significantly reduced in both the LPS and lipid A after irradiation. This is also true for KDO in the LPS. The reduction ranges from 20 up to a maximum of about 60%. The same is true for the sugars in the LPS (Table II) with over 50% of the galactose and glucose being destroyed by irradiation. Table III shows that the destruction of fatty acids in the LPS after irradiation is negligible, in lipid A, however, about 25% of the hydroxy fatty acid is lost.

Table I

The effect of irradiation on glucosamine and phosphate content in LPS and Lipid A, and on the KDO content of LPS

	Glucosamine		Phosphate		KDO	
	nM/mg	%	nM/mg	%	nM/mg	%
LPS	876	100	865	100	141	100
LPS _{irr}	332	37	763	88	116	82
Lipid A	1051	100	1108	100		
Lipid A _{irr}	640	60	784	70		

Table II

The effect of irradiation on the content of monosaccharides in LPS

	LPS nM/mg	LPS _{irr} nM/mg	Recovery (%)
Mannose	4	3	73
Galactose	170	62	36
Glucose	424	176	41
Heptose	347	204	58

Table III

Content of fatty acids in the preparations before and after irradiation, nM/mg

	LPS	LPS _{irr}	Lipid A	Lipid A _{irr}
C12 : 0	57	44	342	300
C14 : 0	98	91	470	365
30HC14 : 0	340	341	2242	1672
C16 : 0	29	27	23	57

Possible changes in the serological specificities of the irradiated preparations were tested in the different haemolysis systems. In addition to the lipid A and LPS systems, also the core systems were used for inhibition by the preparations. The results are summarized in Table IV. It is interesting to note that, compared to lipid A, lipid A_{irr} is an equally potent inhibitor. In spite of the loss of constituents in the course of irradiation there seems to be no loss in the serological specificity. The irradiated LPS_{irr}, in contrast, is a weaker inhibitor due to the destruction of O-specific sugars. LPS_{irr} also proved to be a weaker inhibitor in the Ra and Rb systems. Like LPS, it does not inhibit the Re and Rd system, but in the Re system, which is not inhibited by the LPS, LPS_{irr} does inhibit to some degree. One would conclude that through the destruction of LPS sugars Re determinants are unmasked.

Discussion

Endotoxins have been shown to exhibit in vivo both beneficial and toxic effects [1, 2]. The endotoxic principle responsible for these biological activities is lipid A [18]. When endotoxins are subjected to irradiation with ⁶⁰Co gamma,

Table IV

Passive haemolysis inhibition test of LPS and Lipid A and their irradiation products

Inhibitor preparation	Haemolytic systems						
	Lipid A anti-Lipid A	<i>E. coli</i> O89 LPS-anti- <i>E. coli</i> O89	Ra-LPS anti- Ra-LPS	Rb-LPS anti- Rb-LPS	Re-LPS anti- Re-LPS	Rd-LPS anti- Rd-LPS	Re-LPS anti- Re-LPS
LPS	10.00*	0.2	0.6	0.3	>20	>20	>20
LPS _{irr}	5.00	2.2	2.5	2.5	>20	>20	3
Lipid A	0.01	nd	nd	nd	nd	nd	nd
Lipid A _{irr}	0.01	nd	nd	nd	nd	nd	nd

* Values are expressed as μ g of inhibitor causing 50% inhibition of lysis.

Three haemolytic units of antibody were used

nd = not determined

the biological properties are markedly changed [1, 2, 10, 18–20], and early experiments [7, 10, 20] have indicated that irradiation predominantly causes a reduction in toxic properties while beneficial properties are retained. The dose of 150 kGy ^{60}Co gamma irradiation was found to be optimal for obtaining LPS preparations with beneficial effects and decreased toxicity.

The purpose of this investigation was to determine chemical changes occurring in LPS and lipid A as a result of irradiation. Both preparations were irradiated with 150 kGy of ^{60}Co gamma. The preparations were freeze-dried and then analyzed for the constituents comparatively with the original compounds. The LPS came from a smooth organism, *E. coli* O89, so that changes in all LPS regions would be detectable.

Drastic changes in the composition of all part of the LPS as well as in the free lipid A preparation could be observed due to the destruction of constituents. In some cases (i. e., sugars) 50% of the components were undetectable. Nothing can be said at present about the products of degradation, nor is it known whether these products are still linked to the molecule. Previous data on an Re LPS indicate that some LPS molecules are cleaved, since about 20% of the irradiated material was found to be dialysable [7]. In the present investigation about 25% of lipid A and about 40% of LPS became dialysable after irradiation.

The irradiated lipid A still expresses serological specificity when tested as inhibitor in the lipid A system. In contrast, with the LPS a 10-fold decrease in antigenicity is observed when tested in the homologous system. A similar decrease is also observed in heterologous systems, Ra and Rb. A marked increase, however, is seen in the Re system, which is not inhibited by the original, but significantly inhibited by the irradiated products. This seems to indicate that during irradiation some KDO determinants are exposed.

The results of this investigation are in agreement with data obtained previously with LPS of the Re type [7]. They show that appreciable amounts of constituents are destroyed during irradiation, but at present it is not possible to correlate these chemical changes with the observed biological alteration of the products.

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VIRULENCE ASSOCIATED PLASMID AND R-PLASMID CURING IN *YERSINIA ENTEROCOLITICA* BY SOME TRICYCLIC COMPOUNDS*

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The virulence plasmid which confers calcium-dependent growth in *Yersinia enterocolitica* and the R-plasmid of a *Y. enterocolitica* strain were eliminated by several, tricyclic psychopharmacons, e. g. promethazine, imipramine, and (+) and (–)-butaclamol.

The plasmid-curing action of tricyclic psychopharmacons was described [1, 2] in *Escherichia coli* strains. Later, it was pointed out that plasmids of *Salmonella typhi-murium* [3], *Salmonella panama* [4], *Salmonella london* [5], *Staphylococcus aureus* [6] and a cyanobacterium strain (Katalin Csiszár personal communication) can also be eliminated by chlorpromazine, promethazine or imipramine. The main goal of this work was to test whether the plasmid elimination by some tricyclic drugs also works for yersinia plasmids. For this purpose the N-inc. group of plasmids of *Y. enterocolitica* strains was chosen, because this group of plasmids is stable in many hosts and transferable into various strains of *Enterobacteriaceae* [7, 8], together with another plasmid which confers virulence and Ca-dependent growth in yersinia species. It is known that three species of yersinia harbour a related plasmid that is associated with virulence and Ca-dependent in vitro growth at 37 °C [9]. In this report we compare the plasmid elimination effects of promethazine, imipramine and butaclamol stereoisomers on various plasmids of yersinia.

Materials and methods

Bacterial strains. Ca-dependent *Y. enterocolitica* No. 9576 and its independent plasmidless derivative were obtained from Dr. E. Marjai, Public Health Station, Szeged, Hungary. *Y. enterocolitica* JD224 Amp^r was kindly provided by D. J. Dubel, Research Institute of Infectious Diseases, Frederick, Md., USA. *Y. enterocolitica* Y42 PTE201 (N group Sm^r), *Y. enteroco-*

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litica TP247, PTE212 (N group Sm^r, Tc^r), and *Yersinia pseudotuberculosis* Tateishi PTE101 (N group Sm^r) were kindly provided by Professor S. Kimura, Teiko University School of Medicine, Tokyo, Japan.

Chemicals. Promethazine (Pipolphen®), and imipramine (Melipramine®) were produced by the EGIS Pharmaceutical Works, Budapest. The (+)-butaclamol and (–)-butaclamol were obtained from Research Biochemicals Inc., Wayland, Mass., USA.

Culture media. MTY broth and MTY agar were prepared according to Alföldi et al. [10].

The elimination of R-plasmid was carried out as described earlier [1, 2]. An overnight preculture of yersinia strain resistant to streptomycin or kanamycin were diluted 10⁻⁴-fold, and 0.05 ml (about 5 × 10⁸ bacteria) was used to inoculate 5.0 ml MTY. Different concentrations (1–22 × 10⁻⁴ M) of tricyclic compounds were added to the tubes, which were incubated at 37 °C for 24–48 h. From tubes showing growth, various dilutions were prepared and 0.1 ml samples were plated on MTY agar. After incubation at 37 °C for 16 h, colonies were replicated from MTY agar to MTY agar containing the appropriate antibiotics and, after incubation the master plates were compared with replica plates. Plates were incubated at 37 °C for 24 h, then counted for plasmidless and plasmid-carrying bacterial colonies.

Plasmid elimination of calcium-dependent and independent strains of *Y. enterocolitica* in the presence of promethazine. Calcium-dependent (plasmid-carrying) and independent strains were incubated at 28 °C for 24 h simultaneously in MTY broth without Ca⁺⁺. Samples were removed from the tubes after 24 h, then diluted and plated onto nutrient agar plates. One set of plates was incubated for 48 h at 28 °C (to determine the number of dependent plus independent cells) and another set at 37 °C (to determine the number of cells) that became Ca-independent because of plasmid elimination. The colonies were counted to quantify the plasmid-carrying and plasmidless cells in the population.

Results

The MIC values of promethazine was determined on both Ca-dependent and independent yersinia strains at 28 °C and 37 °C growth in the presence of Ca⁺⁺ 10⁻⁴ M. The Ca-dependent strains were more sensitive than the independent one to the antibacterial action of promethazine at 37 °C. The MIC for the Ca-dependent strain at 28 °C was 160 µg/ml, while at 37 °C it was 120 µg/ml. On the other hand, the MIC for the Ca-independent strain was 160 µg/ml at both temperatures.

The number of colonies grown on the plates at 28 °C involved the plasmid-carrying plus the plasmidless (calcium-independent) cells together, but the number of colonies grown on the plates at 37 °C represented only the plasmidless bacteria (which became calcium-independent) of the population. The ratio of the calcium-independent and dependent cells is the ratio of the plasmidless and plasmid-carrying bacteria in the culture.

The increasing ratio between the calcium-independent and dependent cells indicates the plasmid elimination, which depends on the concentration of promethazine (Table I). Plasmid elimination was fairly effective but the spontaneous loss of this large plasmid (which encoded calcium-binding protein) was also high even without promethazine. The mechanism of elimination can be explained with the existence of plasmid encoded calcium binding protein, which makes sensitive the yersinia to the bactericidal effect of promethazine (Fig. 1) and imipramine (Fig. 2). If bacteria were grown at 37 °C where this protein is synthesized, the promethazine killed the cells more effectively than

Table I

Growth of calcium-dependent strain of *Y. enterocolitica* in the presence of promethazine at 28 °C

Concentration of promethazine $\mu\text{g/ml}$	No. of plasmid-carrying colonies at 28 °C $\times 10^6$	No. of plasmid-less colonies at 37 °C $\times 10^4$	Plasmid elimination (calcium-independent/dependent ratio)
0	615	190	0.00308
60	560	170	0.00303
80	360	140	0.00388
100	170	120	0.00705
120	85	90	0.01058
140	12	18	0.01058
160	—	—	—

those grown at 25 °C where the plasmid encoded protein was missing. In other experiments we tested the plasmid-elimination effects of promethazine and other tricyclic compounds on several yersinia strains (Table II). As the results show, promethazine, imipramine (+)-butaclamol and (–)-butaclamol were able to eliminate the antibiotic-resistance plasmid from *Y. enterocolitica* Y42 PTE201. The R-plasmid of *Y. enterocolitica* TP247 PTE212, *Y. enterocolitica* JD224 and

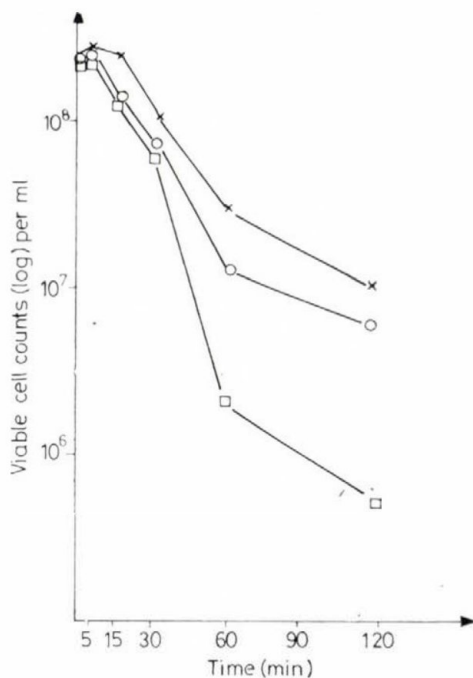


Fig. 1. Bactericidal effect of 6×10^{-4} M promethazine on cured derivative (X—X), and plasmid carrying strain of *Y. enterocolitica* at 25 °C (O—O) and 37 °C (□—□)

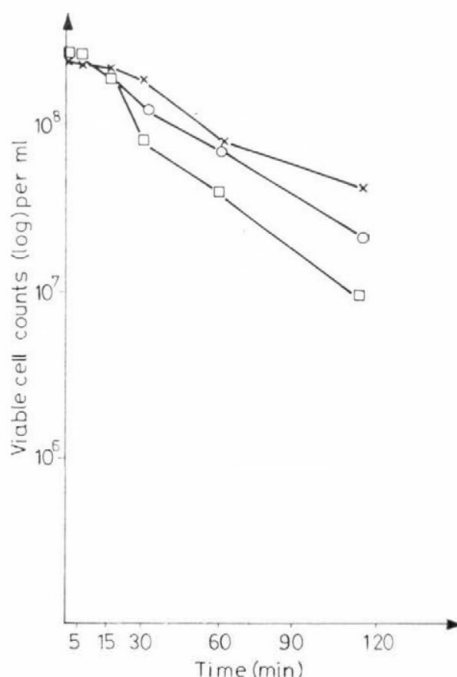


Fig. 2. Bactericidal effect of 1.2×10^{-3} M imipramine on cured derivative (X—X) and plasmid carrying strain of *Y. enterocolitica* at 25 °C (O—O) and 37 °C (□—□)

Y. pseudotuberculosis PTE101 were not eliminable in the presence of tricyclic compounds.

It was interesting that (+)-butaclamol and (–)-butaclamol displayed similar inhibitions of the plasmid replication which shows that the binding of this compounds is not stereoselective in yersinia bacteria. It can be stated that plasmid replication in yersinia strains can be inhibited by several tricyclic psychopharmacoins.

Discussion

Y. enterocolitica exhibits Ca-dependent growth in vitro at 37 °C, but not at 25 °C. A few years ago it was demonstrated by Portnoy et al. [9] that the virulence and expression of Ca-dependence are associated with a plasmid while loss of the plasmid results in Ca-independent growth and avirulence [11]. Recently it has been recognized that Ca-dependence is essential for virulence [12, 13].

Y. enterocolitica expresses at least three novel plasmid-associated polypeptides during growth in nutrient broth at 37 °C [12], but the exact roles of

Table II

Plasmid elimination of Y. enterocolitica and Y. pseudotuberculosis in the presence of tricyclic psychopharmacoins

Compounds concentration $\mu\text{g/ml}$	Plasmid elimination in percent from strains			
	<i>Y. enterocolitica</i>			<i>Y. pseudo-</i> <i>tuberculosis</i>
	JD224	Y42 PTD201	TPA247— —PTE212	PTE101
Promethazine				
40	0	0	0.3	0
60	0	0.5	0.4	0
80	0	1.5	0	0
100	MIC	MIC	MIC	0
120	—	—	—	MIC
Imipramine				
100	0	0.5	0	0
120	0	0.5	0	0
140	MIC	MIC	0	0
160	—	—	0	0
180	—	—	MIC	MIC
(—)-Butaclamol				
100	0	0.5	0	0
120	0	2.0	0	0
140	0	3.2	0	0
160	MIC	MIC	0	MIC
180	0	—	0	—
200	0	—	MIC	—
(+) -Butaclamol				
80	0	0.5	0	0
100	0	0.7	0	0
120	0	1.6	0	0
140	0	0.2	0	0
160	MIC	0.1	0	MIC
180	—	MIC	0	—
200	—	—	MIC	—

these outer membrane proteins are still unknown. They play a part in serum resistance [14, 15] and autoagglutination capacity [16].

There are also plasmids which confer antibiotic resistance in various *Yersinia* species [17] and some of the plasmids in yersiniae were conjugative [18]. The virulence plasmid-encoded Ca-requirement for growth at 37 °C was essential for *Y. pestis*, too, in which loss of this plasmid resulted in a phenotypic expression from virulence to avirulence [19].

The plasmid-elimination effects of the tricyclic psychopharmacoins are theoretically important, because they are able to cure the antibiotic resistance with very low frequency, and with slightly higher frequency the plasmid which

confers the Ca-dependency and virulence on the bacteria. It seems to be that the high concentration of phenothiazines potentiate the calcium deficiency for virulent cells and also promote the growth of avirulent cells. Imipramine and (+)-butaclamol were also effective in plasmid curing but they were less effective than the other two on bacteria. It was theoretically important that (—)-stereoisomer of butaclamol which was inactive in the CNS also cured yersinia plasmids.

It was interesting that the Ca-dependent *Y. enterocolitica* strain growing in the presence of Ca at 37 °C was more sensitive to promethazine than at 25 °C. The plasmid was also lost at 28 °C from the calcium-dependent strain.

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EPIDEMIOLOGICAL PATTERNS AND INCIDENCE OF BIO-, SERO- AND PHAGE TYPES OF *VIBRIO CHOLERA*E IN HYDERABAD, INDIA, DURING 1971–1984

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During 1971–1984 out of 44 762 gastroenteritis cases bacteriologically examined, 4240 (9.5%) *Vibrio cholerae* were isolated in Hyderabad, India. Out of them 1329 (31.3%) were classical and 2911 (68.7%) were el-tor biotypes. The changeover from classical to el-tor cholera was observed in Hyderabad during 1975 and persisted. During this 14 year period four major outbreaks with *V. cholerae* serogroup O1 Ogawa, *V. cholerae* biotype el-tor serogroup O1 Ogawa were observed. Phage types 1, 2 and 4 were prevalent during the classical period and types 2 and 4 during the el-tor period. Of the cholera patients, 67% were under 30 years of age with no significant difference in the incidence among females (51.4%) and males (48.6%). The outbreaks were of protracted pattern with only few cases per day or week with peak incidence during monsoon (May–August). Community of the low socio-economic strata was the most susceptible group. Five zones situated at the outlet of the Moosi river in the downhill were recorded as extensive cholera transmission zones and two of them were the primary foci of infection. The severity of the infection was found directly related to the average rainfall during the year leading to the sewage stagnation in the downhill.

Cholera is still considered as the most important diarrhoeal disease in the South-East Asia, with an unpredictable epidemiological situation. Epidemics in this region during the last three decades emphasizes the emergence of *Vibrio cholerae* biotype el-tor serogroup O1 Ogawa [1–3]. Cholera has been on the offensive after its appearance in the north Celebes and other parts of Indonesia in 1960, during August 1961, passed to Hongkong and into Philippines by September. During 1962–64, el-tor cholera pandemic spread extensively over all the South-East Asian countries passing through India, Pakistan, Afghanistan, reached Iran in 1965 and Iraq in 1966 and in 1970 it spread to Israel, Turkey and USSR and westward into Africa and South Europe [4, 5]. Outbreak of cholera due to toxigenic *V. cholerae* el-tor Inaba was reported from Texas, proved to be the largest outbreak of cholera in the United States in over a century [6]. Thus el-tor cholera shows a tendency to surpass the geographical barriers and unlike classical cholera becomes established in the penetrated areas [5, 7, 8].

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In India, the first case of el-tor cholera was reported by Barua et al., in Calcutta during April, 1964 [9]. Although the classical vibrio was almost completely replaced by el-tor biotype by 1967 in about 10 of the Indian states [1], this variation was observed in Hyderabad, State of Andhra Pradesh only from 1975.

During 1971–1984 a total of 1329 classical and 2911 el-tor biotypes of *V. cholerae* strains were isolated, from Hyderabad, India, with four major cholera outbreaks, in 1973 by classical vibrio and in 1976, 1977 and 1978 by el-tor vibrio. The disease persisted in the form of local endemics with epidemic recrudescence in the rainy season.

This study reports the prevalence of sero and phage types, seasonal patterns, epidemiological characteristics of classical and el-tor cholera during 1971–84 in Hyderabad, India.

Materials and methods

Patients. A total of 44 762 gastroenteritis patients admitted into the Infectious Diseases Hospital, Hyderabad, India, during 1971–84 were examined for *V. cholerae* aetiology. Rectal swabs of the hospitalized patients were collected during the early course of the disease before administration of antimicrobial drugs. Specimens were transported in Venkatraman-Ramakrishnan medium, plated on bile salt agar and/or TCBS agar.

Identification of *V. cholerae* was done according to the standard procedure [10, 11]. The isolates produced oxidase, lysine and ornithine decarboxylase but not arginine dehydrolase, fermented glucose without gas production. The strains were confirmed by agglutination with cholera polyvalent sera. Differentiation of *V. cholerae* strains into classical and el-tor biotypes was based upon Voges-Proskauer reaction, haemolysin production, chick RBC agglutination, sensitivity to polymyxin-B and ability to grow in the presence of hexamine [12, 13]. Serotype of the isolates was determined by agglutination with monospecific sera for Inaba and Ogawa obtained from CRI, Kasauli, India. Phage typing of the *V. cholerae* strains was done at the W.H.O. Collaborating centre for Reference and Research on Vibrios, Calcutta, India.

Results and discussion

Out of the 44 762 gastroenteritis cases 4240 (9.5%) were found positive for *V. cholerae*. The yearly incidence of cholera during 1971–84 is shown in Fig. 1. Four major outbreaks of cholera were observed during the years 1973, 1976, 1977 and 1978.

Incidence of bio-, sero- and phage types

Table I shows the distribution of different biotypes, serotypes and phage types of *V. cholerae* isolated during 1971–84.

In the classical period (1971–74), a total of 1089 *V. cholerae* serogroup Ogawa strains were phage typed. During this period phage types 2 and 4 were prevalent. Phage type 1 appeared only until 1973, whereas phage type 3 was never reported. During 1971 and 1974 phage type 2 was found predominant. In 1972 and 1973 phage type 4 became more prevalent. There was a rise in the

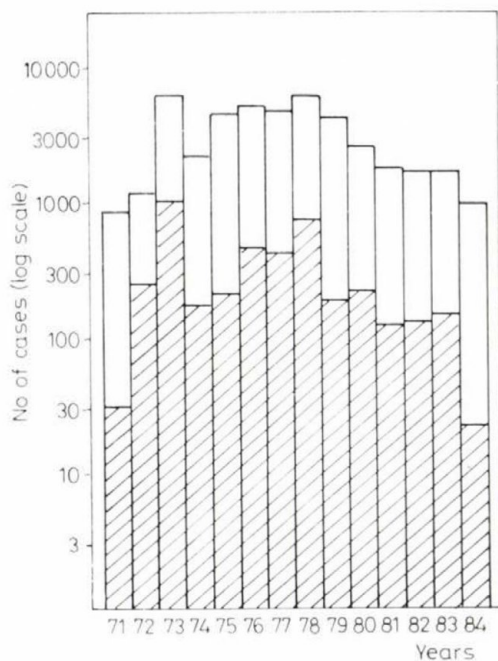


Fig. 1. Incidence of cholera (shaded columns) among gastroenteritis cases (clear columns) during 1971–1984

non O1 vibrio isolations from 1971–73 and during 1974 and later on they were not isolated. The majority of strains typed during the 1973 outbreak belonged to type 4 (83.0%).

In the el-tor period (1975–84) a total of 2198 *V. cholerae* biotype el-tor strains were typed. Both the serogroups Inaba and Ogawa were isolated during the early three years of the el-tor period i. e. 1975 to 1977. Only phage types 2 and 4 were present, among which type 2 was more frequent. From 1978 to 1984, the isolates were *V. cholerae* biotype el-tor serogroup O1 Ogawa. Phage types 2 and 4 were observed. Type 2 was found predominant till 1980, whereas during 1981 both types were prevalent almost with the same frequency, but from 1982 onwards there was a change in the frequency from phage type 2 to 4. Unlike in 1973, during the outbreaks in 1976, 1977 and 1978 strains of phage type 2 were isolated more frequently. Phage types 1 and 3 were never reported during this period. Out of the strains 0.9% were untypable and a total of 50 non-O1 vibrios were isolated during this period. The prevalence and predominance of different phage types during 1971–84 is depicted schematically in Fig. 2.

The Celebes isolates were lysed by all the six phage types and the number of phage types in subsequent outbreaks decreased progressively with time and distance from the original endemic focus in Celebes [14]. As the transmission progressed from primary to secondary foci, so on, finally only two phage types

Table I*Bio-, sero-, and phage types of V. cholerae isolated during 1971-84*

Year	Biotype	Serogroup	Phage type				NAG	NT	Total
			T ₁	T ₂	T ₃	T ₄			
1971	classical	Ogawa	2	22	—	3	4	—	31
1972	classical	Ogawa	22	4	—	164	6	—	196
1973	classical	Ogawa	2	109	—	645	21	—	777
1974	classical	Ogawa	—	53	—	32	—	—	85
1975	el-tor	Inaba	—	123	—	7	18	—	190
	el-tor	Ogawa	—	34	—	8	—	—	
1976	el-tor	Inaba	—	277	—	30	—	—	441
	el-tor	Ogawa	—	176	—	8	—	—	
1977	el-tor	Inaba	—	31	—	7	—	3	427
	el-tor	Ogawa	—	213	—	58	10	6	
1978	el-tor	Ogawa	—	346	—	115	9	2	472
1979	el-tor	Ogawa	—	79	—	8	12	4	103
1980	el-tor	Ogawa	—	116	—	82	—	3	201
1981	el-tor	Ogawa	—	66	—	60	—	1	127
1982	el-tor	Ogawa	—	12	—	85	—	—	97
1983	el-tor	Ogawa	—	37	—	79	1	—	117
1984	el-tor	Ogawa	—	3	—	20	—	—	23
Total			26	1750	—	1411	81	19	3287

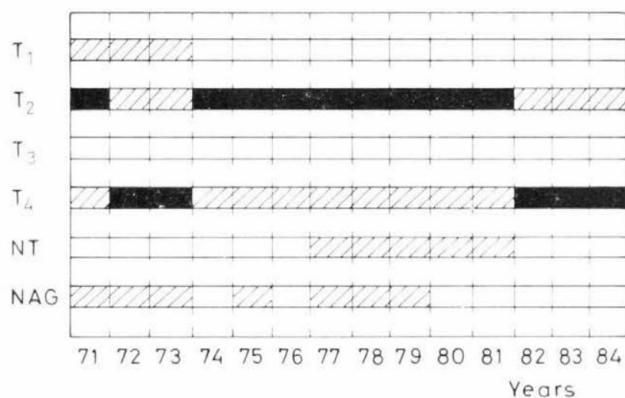


Fig. 2. Phage lytic patterns of *V. cholerae* during 1971-84; clear, absent; shaded, present; solid, predominant

of vibrios were predominantly found in Pakistan and India, where el-tor had entered during 1963-64 [14, 15]. The prevalence of one phage type, either 2 or 4, and occasionally both was observed in the newer countries where biotype el-tor had entered [7].

Until 1966, phage types 2 and 4 were present in India, but in 1967 phage types 2,4 together with type 5, became more prevalent. In 1968, the situation again changed to phage types 2 and 4. It may be noted that in 1967 only seven strains of phage type 5 were reported from 3 Indian states, while the frequency rate increased in 1969 with 52 isolations in 7 Indian states [3], but in 1970 there was not a single isolation of this type. Types 1, 3 and 6 appeared in India in 1969 in a very small number. By 1970, incidence of strains belonging to phage type 1 and 3 had increased but type 6 was missing [7]. This phage typing pattern of the subcontinent markedly reflects in the present investigation.

Age and sex incidence

It may be inferred from Table II that 67% of the cholera incidence during this 14 year study was within 30 years of age and the 21-30 age group being more prone to cholera (24.7%) followed by age group of below 10 years (23.1%) and 11-20 year (19.3%) age group. This situation remained constant during both classical and el-tor periods.

Investigating the cholera incidence in Cairo, it was found that the rate in adults was much higher [16]. According to Pollitzer [17] cholera is most rampant among adults within the age group of 20-50 years. Dizon [18] had shown that cholera was characteristically a disease of adults over the age of 20. In Bangladesh, over 50% of all cholera occurs in the age group 0-9 [19] and the attack rate was over 10 times greater in young children than in adults over 30 years age [20].

Sexwise distribution of cholera cases is shown in Table III. There was no significant difference in the incidence of either classical or el-tor cholera in female (51.4%) and male (48.6%) patients.

John Snow in his early findings during 1850, observed that males were more susceptible to cholera than females [21]. Similar observations were made in Taiwan [22]. In a study at rural Bangladesh it was found that male children were more commonly affected than female children, while adult females had higher rates than adult males [20]. This finding was later supported by Khan et al. [19]. Martin et al. [23] found that these rates were approximately equal for both the sexes. According to Pollitzer, it is very difficult to evaluate the comparative frequency with which cholera occurs in two sexes and in various age groups. Therefore, the difference of opinion by various workers is probably due to different situations prevailing in the area of investigation.

Table II
Age-wise distribution of cholera cases during 1971-84

Year	Below 10 yrs	11-20 yrs	21-30 yrs	31-40 yrs	41-50 yrs	above 50 yrs	Total
1971	1(3.13)	7(21.87)	9(28.12)	8(25.0)	5(15.63)	2(6.25)	32
1972	39(15.18)	34(13.23)	63(24.51)	42(16.34)	41(15.95)	38(14.79)	257
1973	208(20.0)	198(19.04)	254(24.42)	153(14.71)	99(9.52)	128(12.31)	1040
1974	22(12.94)	19(11.18)	96(56.47)	14(8.24)	8(4.70)	11(6.47)	170
1975	58(26.48)	31(14.16)	54(24.66)	23(10.50)	26(11.87)	27(12.33)	219
1976	114(23.70)	95(19.75)	110(22.87)	75(15.59)	43(8.94)	44(9.15)	481
1977	113(26.46)	80(18.74)	105(24.59)	51(11.94)	40(9.37)	38(8.90)	427
1978	226(29.89)	195(25.79)	142(18.78)	108(14.29)	53(7.02)	32(4.23)	756
1979	40(20.72)	45(23.32)	46(23.83)	17(8.81)	18(9.33)	27(13.99)	193
1980	64(27.46)	31(13.31)	58(24.89)	31(13.31)	26(11.16)	23(9.87)	233
1981	16(12.60)	30(23.62)	34(26.77)	20(15.75)	17(13.39)	10(7.87)	127
1982	28(20.89)	18(13.43)	37(27.61)	22(16.42)	9(6.72)	20(14.93)	134
1983	40(27.03)	29(19.59)	38(25.68)	21(14.19)	11(7.43)	9(6.08)	148
1984	10(43.48)	6(26.08)	1(4.35)	2(8.70)	1(4.35)	3(13.04)	23
Total	979(23.09)	818(19.29)	1047(24.69)	587(13.85)	397(9.36)	412(9.72)	4240

Percentage of the cases is shown in parentheses

Table III
Sex-wise distribution of cholera cases during 1971-84

Year	Male		Female		Total
	No.	%	No.	%	
1971	13	40.63	19	59.37	32
1972	120	46.69	137	53.31	257
1973	509	48.94	531	51.06	1040
1974	91	53.53	79	46.47	170
1975	94	42.92	125	57.08	219
1976	220	45.74	261	54.26	481
1977	189	44.26	238	55.74	427
1978	399	52.78	357	47.22	756
1979	105	54.40	88	45.60	193
1980	104	44.64	129	55.36	233
1981	69	54.33	58	45.67	127
1982	56	41.79	78	58.21	134
1983	77	52.03	77	47.97	148
1984	16	69.57	7	30.43	23
Total	2062	48.64	2178	51.36	4240

Seasonal pattern

It may be observed from Fig. 3 that the seasonal incidence of cholera appears to rise at the end of summer, i. e. May and gradually reaches the peak period during monsoon i. e. July and subsides by August. This pattern has been observed both during classical and el-tor periods.

Cholera presents characteristic seasonal variations in endemic regions [8]. In Dacca, cholera appears after the monsoon and disappears in the hot dry

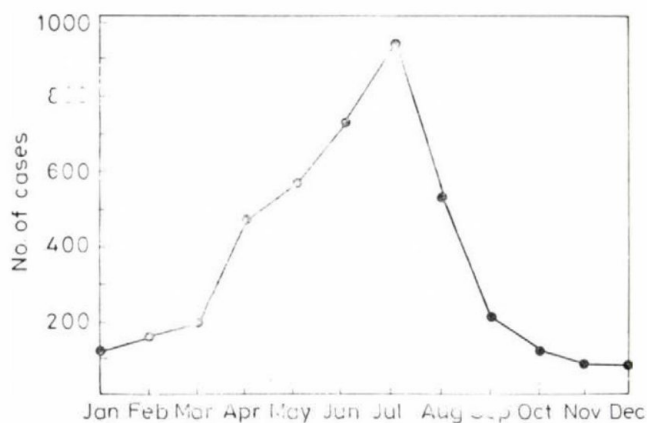


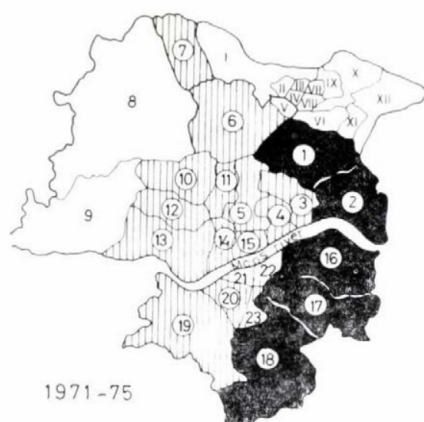
Fig. 3. Seasonal variation (monthwise distribution) of cholera cases

season i. e. November to January [20, 23]. Whereas in Calcutta, the disease reaches a peak in hot dry season and decreases before monsoon i. e. March to June [24]. In the Philippines, the cholera season in certain areas tend to reach its peak with the rainy season, the cause of this seasonal pattern is unknown [25]. At any time of the year, cholera may be initially introduced into an uninfected area, but once established, seasonal recurrences are common.

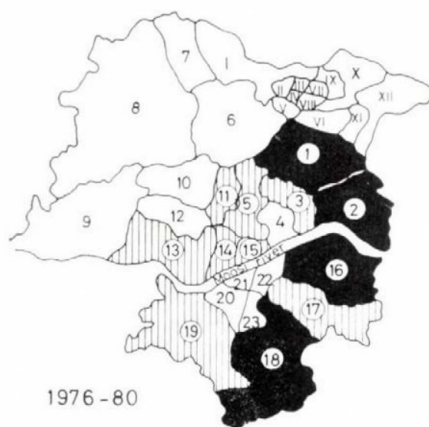
Spectrum of disease transmission

Hyderabad is the capital city for the state of Andhra Pradesh, India, with an area of 65 sq. miles and with 2.65 million population (1981 Census). The city is divided into 23 zones and Secunderabad with the river Moosi intersecting intersecting the city (Fig. 4).

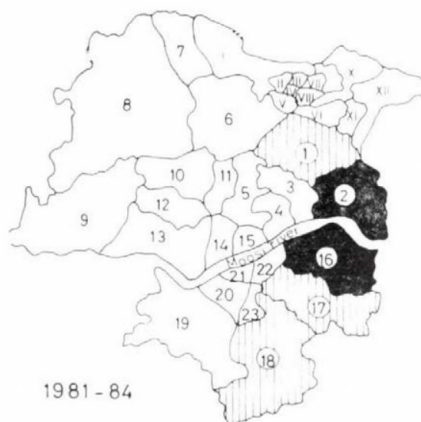
During 1971-75, zones 1, 2, 16, 17 and 18 were found to be severaly affected with cholera and in the neighbouring sixteen zones the incidence was moderate. Zones 8, and 9 and the Secunderabad area were found to be free or inapparent for cholera.



1971-75



1976-80



1981-84

Fig. 4. Changes in the spectrum of cholera transmission and persistence of endemic zones in Hyderabad. Clear, absent; shaded, present; solid, predominant

During 1976–80, in four zones (1, 2, 16 and 18) cholera cases were frequent and only eight of the neighbouring zones were moderately affected.

During 1980–84, the situation considerably improved, but zones 2 and 16 still remained as zones highly prone to cholera, whereas the susceptibility of the neighbouring zones to cholera reduced to three.

Factors contributing to the repeated outbreaks

Cholera epidemics in Hyderabad occur in a protracted pattern with only a few cases per day or week during May–August almost every year. In such outbreaks the route of transmission is not always well defined.

It may be observed from Fig. 4 that the belt of zones, posing the high risk of cholera susceptibility and transmission are at the outlet of the Moosi river. During the monsoon the flow of the flooding water accumulates in the low level outlet of the river, then by causing back flow in sewage disposal systems resulting into stagnation of sewage, particularly in zones 2 and 16. Hence the zones at the uphill of the river are least or not affected by cholera. The cholera incidence has reduced during 1980–84, since rainfall during this period was considerably low.

Usual reservoirs such as river, tank or canal expose a community to a relatively low dose, which occasionally reaches a susceptible person to produce an authentic cholera. Investigations in such communities often reveal numerous inapparent infections and occasionally small explosive outbreaks in family groups exposed to a common food and water supply [25].

Belt of five zones prone to extensive transmission of cholera are situated in the old city, with unsatisfactory sewage disposal system. These are densely populated, with community of low socio-economic status and dwelling in slum environment. Transmission of the disease in this belt may take place through any unhygienic practice via contaminated water, food or by vectors such as flies. Thus persons in the endemic areas whose activities are in close association with water, particularly those in lower socio-economic group, seem to be most susceptible to cholera.

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PRIMING OF *STAPHYLOCOCCUS AUREUS*-INDUCED INTERFERON PRODUCTION IN HUMAN BUFFY COAT LEUKOCYTES BY HUMAN INTERFERON-ALPHA PRETREATMENT

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(Received October 26, 1987)

Human buffy coat leukocytes produced interferon (IFN) in response to induction with heat-killed *Staphylococcus aureus*. Pretreatment of cell with 150 IU/ml human interferon-alpha (HuIFN-alpha) for 3 h enhanced the IFN production by about four- to eight-fold. The simultaneous presence of HuIFN-alpha and gamma in the culture supernatants was indicated by parallel titrations on human embryo fibroblasts (HEF) and MadinDarby bovine kidney (MDBK) cells and also by the distribution of the peak IFN activities after Sephacryl S-200 gel filtration.

Leukocytes can be induced to produce IFN by different stimuli, including bacteria [1, 2]. It has also been reported that pretreatment of cells with homologous IFN primes subsequent IFN production [3]. This priming effect of IFN has been studied mainly on virus or on poly I : C-induced IFN production [1, 4]. Since the enhancement of bacterially induced IFN production can be important in respect to the effectivity of host defense mechanisms, in the present study we have investigated the effect of IFN pretreatment on the IFN production of human leukocytes stimulated with heat-killed *Staphylococcus aureus*.

Materials and methods

Cells and culture media. Human buffy coat leukocytes (HBL) were isolated by lysis of red blood cells with 0.83% ammonium chloride solution [5]. Cells were resuspended at a concentration of 2.5×10^7 /ml in Eagle's MEM containing 2 mg/ml gammaglobulin-free human serum.

Ten hour cultures of *S. aureus* grown in brain heart infusion broth (Difco) were heat-killed by incubation at 90 °C for 10 min. The bacteria were washed twice with PBS and diluted to 1×10^9 microorganisms per ml. For the induction of IFN, a one-tenth volume of this *S. aureus* suspension was added to the HBL cultures and incubated at 37 °C for 15 h.

When the effect of IFN pretreatment was investigated, 150 IU/ml partially purified HuIFN-alpha (spec. act. 2×10^6 IU/ml protein) was added to the HBL cultures. After 3 h incubation, cells were washed three times to remove residual IFN and the inducer was added.

IFN assay. A micromethod based on the inhibition of the cytopathogenic effect of Vesicular Stomatitis Virus was used [6]. Antiviral activity was titrated on monolayers of HEF susceptible to both HuIFN-alpha and gamma and on MDBK cells susceptible to HuIFN-alpha but resistant to HuIFN-gamma. The IFN activities were calibrated against international reference standards HuIFN-alpha (G-023-901-527) and HuIFN-gamma (Gg-23-901-530) and titres are expressed in international units.

Gel filtration was performed on a 2 × 50 cm Sephacryl S-200 column calibrated with bovine serum albumin (BSA), ovalbumin (OA), cytochrome C (CC) and soybean trypsin inhibitor (SBTI) as molecular weight markers.

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Results

Culture supernatants of *S. aureus*-induced HBL cultures exhibited antiviral activities on both HEF and MDBK cells. Titres on HEF were about one order of magnitude higher, which could be a result of the synergistic effects of IFN- α and gamma [7, 8]. Pretreatment of HBL with 150 IU/ml of HuIFN- α for 3 h enhanced these activities in average by more than five times (Table I). Gel filtration analysis of the preparations exhibited peak activities at 21 kD and 58 kD when assayed on HEF, and at 21 kD on MDBK cells (Fig. 1).

Table I

IFN production of primed and unprimed human buffy coat leukocytes induced by heat-killed S. aureus

Priming*	IFN titre** (IU/ml) as assayed on	
	HEL	MDBK
—	192	15
+	1382	118

* 150 IU/ml HuIFN- α for 3 h

** Mean values of three independent experiments each assay of which was performed in duplicate

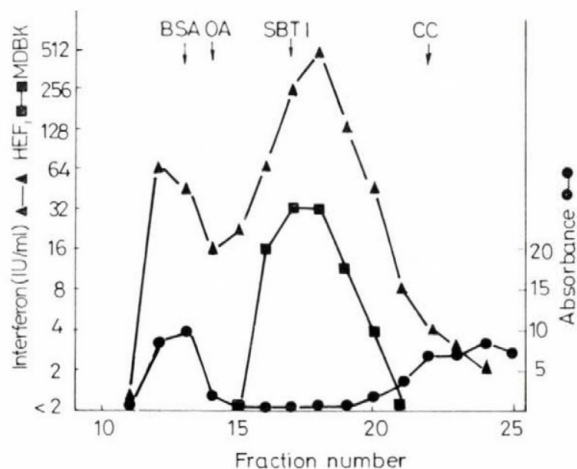


Fig. 1. Gel filtration of *S. aureus*-induced human leukocyte IFN as assayed on HEF and MDBK cells

Discussion

Different IFN types usually have different cellular sources [9]. The interactions of bacteria with HBL therefore seem to involve more than one type of cell which can explain the simultaneous production of different IFN species under these conditions [10, 11]. The presence of lymphokines or other leukocyte products might also be expected as cellular components and enterotoxins of staphylococci are potent mitogens for T cells [12, 13]. Indeed, our previous experiments indicated that culture media of *S. aureus*-stimulated human mononuclear cells supports the growth of an IL-2-dependent murine CTLL cell line [14]. Despite the fact that the significance of IFN production during bacterial infections needs further elucidation, the activation of macrophages by the IFNs produced, and especially by the preponderance of IFN-gamma [15–18], can be an important factor in host defence against invading bacteria.

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CONTENTS

The Aminothiazol-Oxyimino Cephalosporin Antibiotics with Quaternary Ammonium Substituents at the 3-Position (A Review) <i>Uri, J. V., Burdash, N., Bendas, C. M.</i>	327
Therapy of AIDS Patients — Classical Strategies and Proposals for Novel Therapeutic Approaches (A Short Review) <i>Minárovits, J., Berencsi, G., Nász, I., Földes, I.</i>	361
Production of Amylolytic Enzymes in Culture by <i>Botryodiplodia theobromae</i> and <i>Sclerotium rolfsii</i> Associated with the Corm Rots of <i>Colocasia esculenta</i> <i>Nuefo, M. I., Fajola, A. O.</i>	371
<i>Staphylococcus aureus</i> and <i>Staphylococcus epidermidis</i> Strains Differ in Interleukin 2 Inducing Activity <i>Nemes Nagy, Zs., Rosztóczy, I.</i>	379
Long-Term Preservation of the Enteroinvasive <i>Escherichia coli</i> Factor Responsible for Experimental Keratoconjunctivitis <i>Šourek, J., Aldová, E.</i>	383
Feeding by Mucin and Intestinal Growth of Some Enteric Bacterial Pathogens <i>Kétyi, I.</i>	389
Properties of Interferons Produced by Different Cell Populations <i>Nossik, N. N., Bopegamage, S. A., Yershov, F. I.</i>	397
Effects of Interferon and Tumour Necrosis Factor on Development of Intracellular Bacterial Infections <i>Degré, M., Bukholm, G.</i>	405
Inhibited Interferon Production After Space Flight <i>Sonnenfeld, G., Gould, C. L., Williams, J. A., Mandel, A. D.</i>	411
Antigenic Structure of Mammalian Adenoviruses Studied by Monoclonal Antibodies Against Bovine Adenovirus Type 2 <i>Rusvai, M., Belák, S., Stolz, B., Ádám, Y., Nász, I.</i>	417
Characterization of Multiply Resistant <i>Proteus mirabilis</i> Isolates in Hungary <i>Konkoly Thege, M., Nikolnikov, S.</i>	423
Effect of Herbicides on Growth of Some Microscopic Soil Fungus Species <i>Helmeczi, B., Kátai, J., Bessenyei, M.</i>	429

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THE AMINOTHIAZOL-OXYIMINO CEPHALOSPORIN ANTIBIOTICS WITH QUATERNARY AMMONIUM SUBSTITUENTS AT THE 3-POSITION

(A REVIEW)

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(Received March 25, 1988)

Introduction and grouping of cephalosporins	327
Synthesis and significance of the aminothiazol side chain	329
The origin of the oxyimino-aminothiazol acetamido cephalosporins	329
Overview of the aminothiazol-syn-oxyimino cephalosporin antibiotics	331
The aminothiazol-oxyimino side chain attached to non-cephalosporin beta-lactams	332
Aminothiazol syn-oxyimino cephalosporins with heterocyclic quaternized ring substituents at the 3-position	333
General characteristics	333
The individual compounds	335
Ceftazidime	336
Cefpirome	338
Cefepime	342
DN-9550	346
DQ-2556	347
BO-1236	348
ICI-194008 and ICI-193428	349
BO-1232, CM-40874, GR-32620 and an unnamed isoquinoline	350
Non-aminothiazol cephalosporins with 3-quaternary substituents	353
Cephaloridine	353
Cefsulodin	353
Cefpimizole	354
Concluding remarks	354
References	355

Introduction and grouping of cephalosporins

The cephalosporins, belonging to the larger family of beta-lactam antibiotics, occupy an especially distinguished position among all of the highly successful antibacterial chemotherapeutic agents. The reason for such a position is that these compounds are extremely effective in combating bacterial infections and are, at the same time, very safe and devoid of any significant adverse effects. Today, the cephalosporins represent one, if not the most, important group of antibiotics employed against infectious diseases of bacterial origin. These compounds have been claimed to be not only scientific but also medical successes and have contributed to saving lives and restoring the health

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of millions of patients. They are all semisynthetic derivatives of 7-aminocephalosporanic acid (7-ACA), the common nucleus of the cephalosporins, collectively referred to as cephems.

Today, about twenty-five years after the introduction of the first cephalosporin product into human therapy of infectious disease, the number of cephalosporins used in various parts of the world approaches three dozen. It is customary to group these compounds into "generations" on the basis of their antibacterial spectra, susceptibility or resistance to bacterial beta-lactamases (deactivating enzymes) of diverse origin, the depth of potency and efficacy, as well as, their pharmacokinetic properties. Since these parameters are apparently structure-dependent, the chemical substituents on the cephalosporin nucleus (7-ACA) may be also considered in the classification of the cephalosporins. Without further characterization and with the note that there are occasional borderline products, the list of approved cephalosporins within their corresponding generation is given as follows.

1. *First-generation cephalosporins*: cephalothin, cephaloridine, cefazolin, cephadrine, cephapirin, cephalixin, cefadroxil, cefrodaxine, cefazedone, ceftezole, cephacetrile.

2. *Second-generation compounds*: cefamandole, cefuroxime, cefonicid, cefatrizine, ceforanide, cefaclor, cefotiam.

3. *Third-generation products*: cefotaxime, cefodizime, ceftiole, cefpirome, cefepime, ceftizoxime, ceftriaxone, cefmenoxime, ceftazidime, ceftoperazone, cefsulodine, cefpimizole, cefpiramide.

4. *Compounds in the subgroup of cephamycins*, such as ceftiole, cefotetan, cefbuperazone, cefmetazole and the oxacephamycin, moxalactam, may be classified as either second- or third-generation cephalosporins.

The first nine drugs listed among the third-generation cephalosporins and many other compounds bearing only code numbers (not listed here), as well as, the second-generation compound, cefotiam, belong to a recently discovered and growing group of cephems, the aminothiazol cephalosporins, which have quickly gained clinical use. With the exception of cefotiam, these compounds also contain a *syn*-methoxyimino, or other generally termed oxyimino group, attached to the C-2, or alpha-position, of the 7-acetyl side chain. These are presently the most potent cephalosporins possessing broader antibacterial spectra and higher degrees of resistance to deactivation (hydrolysis) by most beta-lactamases.

These aminothiazol cephalosporins contain different substituents at the 3-position of the cephem molecule which may modify (improve) the antibacterial activity and particularly the pharmacokinetics of the individual drugs. A small, but constantly increasing in number and importance, subgroup of the aminothiazol oxyimino third-generation compounds are those which contain at the 3-position a polar, aromatic quaternary ammonium ring-

system. Many of these compounds possess additional advantageous properties over the other third-generation compounds. Some of them may deserve to be categorized as „fourth-generation” cephalosporins.

The subject of this paper is to review and discuss the discovery and introduction of the aminothiazolacetyl side chain, the origin of the oxyimino group in the acetyl side chain and to describe, in considerable detail, the earlier and newer 3-quaternary aminothiazol-oxyimino cephalosporins.

Synthesis and significance of the aminothiazol side chain

The synthesis of the aminothiazol side chain was the result of decades of searching in an empirical manner for hetero-acetyl chemical compounds to be attached to the 7-position of the cephalosporanic acid molecule. It was a great and pleasant surprise when, on a purely speculative basis, thiourea in place of thiocyanate was added to the 7-amino-cephalosporanic acid solution and resulted in the condensation of the thiourea to form 2-aminothiazol-4-ylacetyl cephalosporin in good yield [1]. This serendipitous discovery [2] was very successful since its introduction tremendously increased the activity of the parent compound against Gram-negative bacilli. It also introduced a new activity not known with earlier cephalosporins, namely, activity directed against *Pseudomonas aeruginosa*, as is shown in Table I. The figures are taken and modified from reference [2]. The superior activity of the aminothiazol compound (last in the column) is indisputable.

The first cephalosporin introduced into human therapy bearing the unsubstituted aminothiazol acetic acid side-chain at the 7-position was the second-generation drug, cefotiam [3-7]. It possesses a broader spectrum than cefazolin, cefamandole and cefuroxime, and includes activity against *Enterobacter*, *Citrobacter* and *Proteus* spp., *Haemophilus influenzae* and *Neisseria gonorrhoeae*. It is also somewhat more resistant to beta-lactamases.

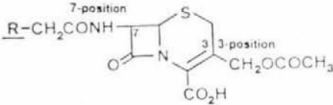
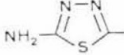
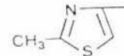
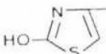
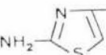
The origin of the oxyimino-aminothiazol acetamido cephalosporins

A significant step in the advancement of the chemistry and biology of the cephalosporins was culminated by the introduction of a methoxyimino function in the alpha-(C2)-position of the acetyl side chain of the aminothiazol group at the 7-position of the cephem nucleus.

The forerunner of this structural entity, the hydroxyimino group at the alpha-position of the acylamino moiety, was originally found in the structure of the natural product, nocardicin A (Fig. 1), which is a monocyclic beta-lactam antibiotic with activity against Gram-negative bacteria including

Table I

In vitro antibacterial activity of cephalosporins with various heterocyclic substituents at the 7-position
Modified from reference [2]

					
MIC (μg/ml)					
R	<i>S. aureus</i>	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>E. cloacae</i>	<i>P. aeruginosa</i>
	0.4	17	2	5	0
	0.8	25	6.2	—	0
	1.6	12.5	12.5	>200	0
	0.8	50	12.5	25	0
	1.6	6.2	6.2	25	0
	1.6	1.6	0.4	0.4	3.2-50

P. aeruginosa [8-11]. As it often happens in the drug development process, this natural product served as the prototype for the synthesis of oxyimino-substituted cephalosporins. The methoxyimino aminothiazole side chain was first used in cefuroxime (Fig. 1). Many of these oxyimino-aminothiazol cephalosporins have proven to possess activities so high and broad as never have been observed before for any of the cephalosporins or other antibiotics [12].

The term "oxyimino" as used throughout this text will cover hydroxyimino, alkoxyimino, alicyclic- and heterocyclic-oxyimino groups found so far in compounds used in therapy or which are in various stages of development.

The origin and development of the combined aminothiazol-oxyimino side chain is shown in Fig. 1. It can be seen that this arises by merging the aminothiazol ring of cefotiam with the *syn*-(Z)-methoxyimino component first found in the structure of cefuroxime [11, 13-15].

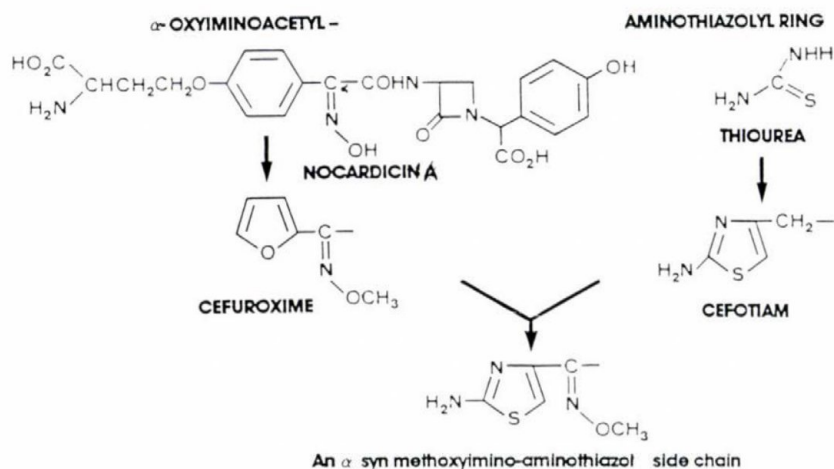


Fig. 1. Origin of the aminothiazol syn-oxyimino side chain

Overview of the aminothiazol syn-oxyimino cephalosporin antibiotics

The early recognition of the extraordinary importance of the introduction of the 2-aminothiazol-4-yl-2(α)-syn-hydroxy- or methoxyimidoacetic acid side chain to the 7(β)-amino-position of the cephalosporanic acid initiated an enormous amount of research in synthesis to modify the oxyimino group and attach various substituents at the 3-position of the beta-lactam dihydrothiazine nucleus. The publications in this area are too numerous to cite completely and only those most closely related to the objective of this review are selected here [1, 3, 11, 12, 16–32].

It was recognized from the beginning of this research, and should be kept in mind, that only the *syn* (Z) variants (geometry) have excellent activity. The *anti* (E) isomers possess much lower, or often negligible, in vitro and in vivo antibacterial activities [3, 11, 33, 34]. Consequently, the correct descriptive naming of these compounds should be: 7- β -[(Z)-2-oxyimino-2-(2-aminothiazol-4-yl)acetamido]-cephalosporins with the addition of the chemical nature of the 3-substituent, such as 3-pyridinium or 3-methylpyrrolidinium in the case of the quaternary compounds which serve as the subject of the remaining portion of this review.

Interestingly enough, the chemical colour reaction developed for these compounds separates the two isomers; only the very active *syn* (Z) isomers yield the cherry red colour, not the *anti* (E) isomers [35]. This high degree of specificity is amazing.

The oxyimino-aminothiazol third-generation cephalosporins possess an extended antibacterial spectrum beyond that of the first- and second-generation compounds. Many of these cephalosporins are effective against *Serratia mar-*

Table II

The first aminothiazol alkoxyimino cephalosporins introduced into human therapy
Modified from reference [28]

Generic name	Company	Year of patent
Cefotaxime	Hoechst-Roussel	1976
Cefmenoxime	Takeda; Roussel	1976
Ceftizoxime	Fujisawa	1977
Ceftazidime	Glaxo	1978
Ceftriaxone	Hoffmann-La Roche	1978

crescens, indole-positive *Proteus* spp., *Enterobacter* spp., *Citrobacter freundii*, *P. aeruginosa* and the anaerobic Gram-negative *Bacteroides fragilis*. They are also resistant to most beta-lactamases to various degrees.

The first aminothiazol-oxymino cephalosporins patented and approved for prescription use are listed in Table II (modified from reference [28]). All of the five listed drugs are excellent third-generation antibacterial cephalosporins with some variations in their spectra, pharmacokinetics and clinical uses. A few remarks about their distinguishing features include the fact that cefotaxime was the first one of these compounds approved for general medical use in the USA. Also, ceftizoxime, developed in the USA by Smith Kline and French Laboratories [36, 37], has been shown to display good activity against *B. fragilis*. Additionally, ceftazidime's anti-pseudomonal activity supersedes that of the potentially toxic aminoglycosides, such as gentamicin. Of those drugs listed in the table, ceftriaxone is the most active against *Neisseria gonorrhoeae* and possesses a long half-life in man (about 8 h).

In addition to those compounds listed in Table II, a large series of newer aminothiazol-oxymino cephalosporins has been described with many of them in various stages of clinical development. The descriptions and detailed discussions of these compounds, especially the older ones, can be found in many review articles [28, 35, 38-47]. For those who wish to be more familiar with the clinical applications, therapeutic doses and eventual adverse effects of these aminothiazol cephalosporins, the following articles, book chapters and reviews will serve as excellent source materials [48-54].

The aminothiazol-oxymino side chain attached to non-cephalosporin beta-lactams

It is interesting to note that the successful 2-aminothiazol-4-ylacetyl oxymino side chains have also been attached to beta-lactam structures other than the cephalosporins. These structures include the oxa- and carbacephem

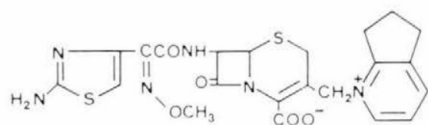
skeletons and the monobactams [31, 55]. These combinations have also yielded therapeutically successful beta-lactam drugs. The monobactams are derivatives of the newly-isolated beta-lactam nucleus referred to as 3-aminomonobactamic acid (3-AMA), the simplest beta-lactam structure. These new compounds include: aztreonam [56], carumonam [57], tigemonam [58] and monobactam BO-1165 [59]. These are very active compounds possessing activity mainly directed against Gram-negative bacilli including *P. aeruginosa*. They are resistant to the common beta-lactamases. Aztreonam has been approved in the USA for the treatment of infections caused by Gram-negative bacilli [60]. Other members of this group are under clinical development. Further discussion of these interesting drug products and drug substances is beyond the scope of this review.

Aminothiazol syn-oxyimino cephalosporins with heterocyclic quaternized ring substituents at the 3-position

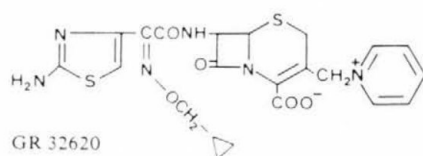
General characteristics

Compounds belonging to this distinct group represent the newest developments in cephalosporin research. They came into existence through a continued and intensive research program which searched for new derivatives to overcome certain shortcomings of the earlier aminothiazol cephalosporins. The research goals included increased activity against *P. aeruginosa* and other pseudomonal species, as well as, increased activity against *Staphylococcus aureus*, and acquisition of activity against *Streptococcus faecalis*, methicillin-resistant *S. aureus*, *S. epidermidis* and *B. fragilis*. With the exception of the last item, the research has been largely successful. For possibly better anti-pseudomonal activity, the results with ceftazidime in this respect, have provided some good prospects. In addition to the above-mentioned items, a novel form of beta-lactamase stability has been observed and utilized.

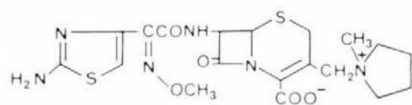
All of these new aminothiazol cephalosporins have the common chemical characteristic that they contain a basic substituent, a quaternary ammonium group, attached to the 3-position of the methylene-dihydrothiazine portion of the cephem nucleus. They are all invariably referred to in the literature as 3-quaternary ammonio cephalosporins, or quaternary cephalosporins. Those described, or mentioned, in the literature to date (by completion of this manuscript in February, 1988) are presented in Fig. 2. Included in this figure is the earliest compound in the group, ceftazidime. It can be seen that all of the compounds are zwitterionic and, with the exception of ceftazidime, they have no net anionic charge, since the negative charge of the carboxylic acid is (internally) neutralized (compensated) by the positive charge of the 3-quater-



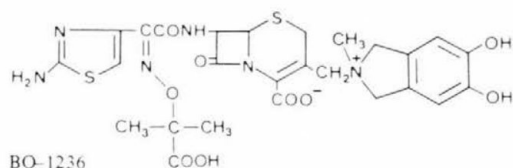
Cefpirome



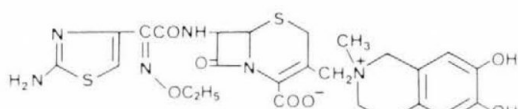
GR 32620



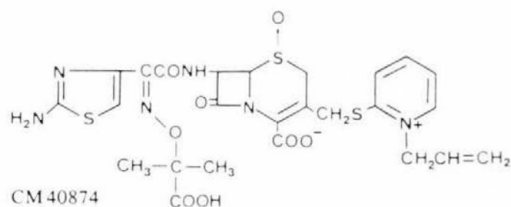
Cefepime



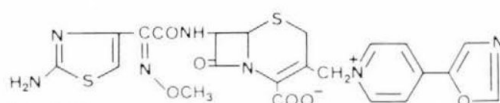
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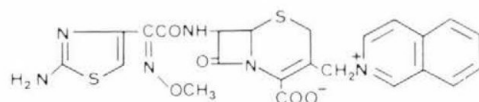
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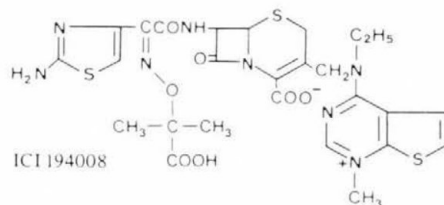
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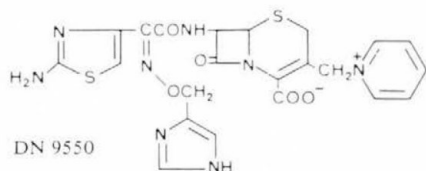
DQ-2556



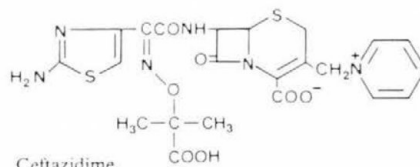
An isoquinoline (no code)



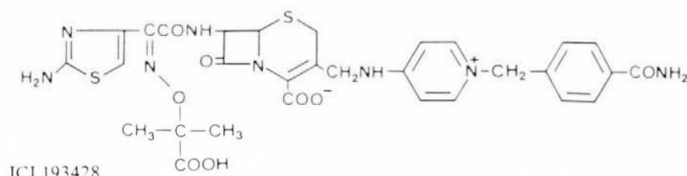
ICI 194008



DN 9550



Ceftazidime



ICI 193428

Fig. 2. Chemical structures of 3-quaternary aminothiazol *syn*-oxyimino cephalosporins

nary ammonium group. This is a betaine. This chemical configuration has been associated with a high water-solubility and easy penetration into the bacterial cell across cytoplasmic membranes probably through porin channels [61].

The quaternary aminothiazol cephalosporins have remarkable biological properties [28, 31, 46, 47, 62, 63]. They have greatly improved activities against staphylococcal and streptococcal strains and are the first cephalosporins which demonstrate activity against methicillin-resistant strains of *S. aureus* with MIC₅₀ of 5.6 µg/ml and MIC₉₉ of 48 µg/ml. Their activity against *P. aeruginosa* is comparable to or better than that of ceftazidime. Also, these compounds are the first cephalosporins with acceptable MIC values (7.0 µg/ml) against *S. faecalis*. Against *Enterobacter*, *Citrobacter* and *Acinetobacter* spp. these quaternary cephalosporins have an improved activity with an MIC₉₀ at about 1.0 µg/ml. Although they are active against Gram-positive anaerobes and non-beta-lactamase-producing *B. fragilis*, the enzyme-producing strains are resistant to them.

The subcutaneous mouse ED₅₀ values of these compounds are in agreement with their MIC values whether the experimental infection was performed with Gram-positive or Gram-negative bacteria. Possibly related to the chemical-structural features, these cephalosporins are highly resistant to the hydrolysis by beta-lactamases of both chromosomal and plasmid origin. Additionally, they have relatively low affinities to the various beta-lactamases, consequently they are not trapped by them in the periplasmic space and therefore evade the non-hydrolytic destruction which would lead to resistance development [64, 65]. These cephalosporins bind predominantly to the penicillin binding protein 3 (PBP3), which explains their excellent anti-pseudomonal activities and their bacteriolytic actions.

On the basis of the above-mentioned chemical and, above all, biological characteristics, it is tempting to classify the new quaternary cephalosporins as "fourth-generation" cephalosporins [66, 67].

The individual compounds

Of the quaternary cephalosporins appearing in the literature today and listed in Fig. 2, only the earlier synthesized and studied aminothiazol-containing compound, ceftazidime, has been approved for general use. The other compounds are in various stages of development. Cefpirome and cefepime are in the most advanced clinical developmental stage. These compounds will be discussed in a considerably detailed fashion whereas the others to only a limited extent. Both categories will be supported by published papers in journals, however, abstracts generally will not be used for reference purposes. Some of the publications referenced in the first part of this summary may list some of the new quaternary cephalosporins along with their brief structure-activity

relationships, but this review is the first attempt to bring together and discuss all of these compounds in a comparative manner. All of their relevant aspects will be discussed including their biological actions and effects which are thus far not found elsewhere in the literature. For comparative purposes, the discussion will begin with ceftazidime which was the first drug in this group of the oxyimino-aminothiazol quaternary cephalosporins. This description will be followed with the review of the newer compounds in the group.

Ceftazidime

Ceftazidime (GR 20263) is the earliest member of the oxyimino-aminothiazol quaternary (C-3 pyridinium-containing) cephalosporins. An older cephalosporin, cephaloridine, also contains the 3-pyridinium ring but possesses a different substituent at the 7-position and will be discussed later. Today, ceftazidime is a well-established, broad spectrum, third-generation (sometimes referred to as a fourth-generation) cephalosporin. In contrast to other members of this group, it contains a carboxypropyloxyimino substituent attached to the alpha(C2)-acetyl chain. In the case of the majority of the aminothiazol cephalosporins, this substituent is a methoxyimino group (see Fig. 2). It is of interest since the additional carboxyl group provides acidic (negative charge) properties to the molecule. The carboxylate anion on the cephem ring is neutralized by the positively charged quaternary ammonium group. The extra negative charge strongly influences the pharmacokinetic profile of ceftazidime, which in general is excellent.

Ceftazidime's rapid acceptance as a first-line anti-pseudomonal cephalosporin is reflected by the thousands of publications about it in the literature. It would be impossible to list all of them in this publication. The list here will be restricted to the first three original papers [68-70], two voluminous books [71, 72], some selected reviews [73-77] and a more comprehensive review [78]. A few recent papers will also be referenced.

Although it is a well-known and widely used drug, a brief review of ceftazidime is in order. Ceftazidime is a parenteral, broad spectrum, beta-lactamase stable, quaternary ammonium cephalosporin. It is active against Gram-negative bacteria, especially members of the family *Enterobacteriaceae*, and, to a somewhat lesser extent, against Gram-positive bacteria. It is resistant to most beta-lactamases and is also active against many beta-lactamase-producing strains. Presently, ceftazidime is the most effective cephalosporin available against *P. aeruginosa*. It is highly effective in infections of the respiratory and urinary tracts caused by susceptible bacteria even in debilitated patients. It is so potent and successful, that in the USA it is marketed by three leading pharmaceutical companies as Fortaz (Glaxo), Tazicef (Smith Kline and French) and Tazidime (Lilly).

In vitro antibacterial activities of ceftazidime cover a large number of human pathogens including *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella*, *Shigella*, *Proteus vulgaris* and indole-positive *Proteus* spp., *Serratia marcescens*, *Providencia* spp., *Citrobacter* spp., (except *C. diversus*), *P. aeruginosa*, *P. cepacia*, *P. alcaligenes*, *P. maltophilia*, *P. putida* (other species of *Pseudomonas* are non-susceptible), *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *N. meningitidis*, and most *Acinetobacter* sp., *Staphylococcus aureus* and *Clostridium perfringens* are moderately susceptible in vitro but in vivo ceftazidime is effective. *B. fragilis*, *C. difficile*, *S. faecalis* and methicillin-resistant *S. aureus* are usually not susceptible to ceftazidime.

In vivo experimental infection-protection studies in mice and other experimental laboratory animals have confirmed the in vitro activities of the drug. The ED₅₀ values in mice were found to be 6.2–34 mg/kg against *S. aureus*, 0.1–3.5 mg/kg against most Gram-negative bacilli and 1.6–4.5 mg/kg against *P. aeruginosa* strains. Recently published is a report of a rare development of ceftazidime resistance during therapy for an *Enterobacter cloacae* infection [79].

Ceftazidime possesses favourable pharmacokinetics. Its binding to plasma proteins is low, ranging from 8–23% and is independent of the serum concentrations. Serum concentrations for ceftazidime are high, immediately after intravenous infusion over 20–30 min of 1.0 g the serum concentration usually is about 70 µg/ml. After a 1 g bolus intravenous injection these values can reach 120–140 µg/ml. One hour after a 1 g intramuscular injection of the drug, the peak serum concentrations achieved range between 37 and 43 µg/ml. Serum levels are dose-dependent. Ceftazidime is eliminated via the urine as intact, non-metabolized drug by glomerular filtration. The elimination half-life ($T_{1/2}$) of ceftazidime is 1.5–2.8 h. In patients with renal impairment and neonates this period is prolonged.

Ceftazidime can be given by intravenous or intramuscular injections twice or three times a day as a divided dose. The adult dose ranges between 1 and 6 g daily depending on the severity of the infection and the susceptibility of the infecting organism. In urinary tract infections, 0.5–1.0 g twice daily is usually adequate. The paediatric dose can range from 30 to 100 mg/kg in two or three divided doses, and the dose for neonates is 25–60 mg/kg. In renal impairment, the doses must be decreased as the creatinine clearance dictates.

Ceftazidime has been shown to be effective in a wide range of bacterial infections in adult, paediatric and neonatal patients. Very good clinical and bacteriological efficacy has been demonstrated in serious Gram-negative infections of the respiratory and urinary tracts, skin, soft tissue and bone. It is also effective in septicaemia, Gram-negative meningitis, gall bladder empyema, obstetric and gynaecological infections, and chronic and recurrent otitis media. It has also been shown to be efficacious in the treatment of *P. aeruginosa* respiratory tract infections in patients with cystic fibrosis, as well

as, in other systemic pseudomonal infections where ceftazidime has virtually replaced the toxicity-prone aminoglycoside antibiotics. It also can be used for the treatment of immuno-compromised patients, usually in combination with other antibiotics to increase the Gram-positive activities.

Ceftazidime is well-tolerated and its side-effects are rare, transient and similar to those observed with other beta-lactam antibiotics. Kidney toxicity, a problem with cephaloridine the first cephalosporin possessing the 3-pyridinium side-chain, has not been observed with ceftazidime. The reason for the success of ceftazidime is the result of the combination of the following favourable features [80]: (a) broad spectrum of activity, including activity against *P. aeruginosa*; (b) high intrinsic activity at the bacterial cell target; (c) efficient penetration into bacterial cells; (d) resistance to bacterial enzyme degradation; (e) efficient absorption; (f) good tissue penetration; (g) negligible adverse side-effects; (h) metabolic stability; (i) low serum protein binding; (j) good pharmacokinetics and patient tolerance. These characteristics make ceftazidime an acceptable replacement for the more toxic aminoglycoside antibiotics. It is also expected that the newer quaternary ammonium cephalosporins, described below, can serve as "improved editions" of ceftazidime.

Cefpirome

Cefpirome is a new, semi-synthetic, parenteral alpha-syn-methoxyimino aminothiazol cephalosporin with a polar cyclopentanopyridinium group at the 3-position (Fig. 2). The sulfate salt of cefpirome, intended for medical use, is a crystalline monohydrate, which is highly soluble in water (zwitterionic) at physiological pH ranges. It is fairly stable in aqueous solution.

Cefpirome has been described as an outstandingly active and very broad-spectrum cephalosporin. Its antibacterial spectrum appears to cover such genera as methicillin-resistant *Staphylococcus* spp., which represent clinical problems with most third-generation cephalosporins.

The in vitro antibacterial activity of cefpirome has been extensively studied using a large number of bacterial isolates [81-86]. Although the studies were done with different techniques, media and strains, the list of strains used and the summary median inhibitory concentrations (MIC's) provide a general impression of the in vitro activity of cefpirome. It has the lowest MIC values (MIC₅₀ and MIC₉₀) among the third-generation cephalosporins against Gram-positive genera such as *Staphylococcus* spp., methicillin-resistant *Staphylococcus* spp. and *Streptococcus* spp. (including enterococci). The corresponding MIC₅₀ and MIC₁₀₀ values for the organisms listed above are, in µg/ml, 0.26 and 1.0, 5.6 and 48.0, < 0.002 and 2.3, and 1.9 and 7.0.

Its potency against Gram-negative bacilli is even greater. MIC₅₀ and MIC₉₀ values (in µg/ml) for the following Gram-negative organisms are 0.005

and 0.038 for *E. coli*, 0.007 and 0.023 for *Salmonella* spp., 0.018 and 0.13 for *Klebsiella* spp., 0.024 and 0.71 for *Enterobacter* spp., 0.101 and 3.8 for *Acinetobacter* spp., 1.6 and 6.1 for *P. aeruginosa*, 0.46 and 115 for other *Pseudomonas* spp., 0.046 and 0.149 for *P. mirabilis*, 0.027 and 0.056 for *P. vulgaris*, 0.149 and 1.36 for *Morganella morganii*, 0.015 and 0.66 for *P. rettgeri*, 0.101 and 0.38 for *Providencia* spp., 0.025 and 0.17 for *Serratia* spp., and 0.013 and 0.19 for *Citrobacter* spp.

Special studies have reported on the in vitro activity of cefpirome against Gram-positive aerobic cocci [87], strict anaerobic bacteria [88] and anti-pseudomonal activities [89]. All methicillin-resistant *S. aureus* strains were inhibited by 1.0 µg/ml, those of *S. epidermidis* by 4.0 µg/ml and the enterococcus strains by 16 µg/ml.

The modal MIC's (in µg/ml) against anaerobic bacteria for cefpirome were as follows: 16 for *B. fragilis*, 128 for *B. thetaiotaomicron*, 0.5 for *B. vulgatus*, 0.25 for *Peptococcus asaccharolyticus*, 0.5 for *P. magnus*, 2.0 for *P. variabilis*, ≥ 32 for *Fusobacterium* or *Veillonella* spp., < 0.12 for *Clostridium perfringens*, 128 for *C. tertium*, ≤ 16 for *C. difficile*, 32 for *Eubacterium lentum*. Many anaerobic strains were found to be more susceptible to cefpirome than to ceftazidime.

In a comparative study with other anti-pseudomonal agents, cefpirome was found to be most active against strains of *P. aeruginosa*, *P. pickettii*, *P. putida*, *P. acidovorans*, *P. fluorescens* and *Acinetobacter calcoaceticus* with MIC₅₀ and MIC₉₀ values of ≤ 4 and ≤ 16 µg/ml, respectively. Against *P. stutzeri* and *P. mendocina*, the MIC₅₀ was ≤ 0.25 µg/ml and the MIC₉₀ was ≤ 1 µg/ml. Cefpirome was found to have an MIC₅₀ of ≤ 8 µg/ml against carbenicillin-resistant *P. aeruginosa* and *A. calcoaceticus* strains. It was, however, not active against other *Pseudomonas* species and other non-fermenters, such as *Flavobacterium* sp.

Cefpirome displays synergistic activity in vitro with the aminoglycosides, such as gentamicin and amikacin, when used against multi-resistant Gram-positive and Gram-negative bacteria [90].

The in vitro activities of cefpirome were favourably compared with five other cephalosporins (cefoperazone, cefotaxime, ceftazidime, ceftriaxone and moxalactam), as well as, two aminoglycosides (gentamicin and amikacin), against a series of Gram-positive and Gram-negative bacteria [91]. In another series, a comparative study was performed against enterococci using teicoplanin, ampicillin, piperacillin, gentamicin and vancomycin. Teicoplanin was the best, and by and large, cefpirome was comparable to the others as for the MIC and MBC values.

The in vitro activity of cefpirome was compared with that of cefepime (the next compound to be discussed in this review), WIN-49375, imipenem, ceftazidime, aztreonam, piperacillin and timentin against 253 nosocomially

important bacterial isolates from cancer patients. The isolated bacteria, *E. coli*, *Klebsiella* spp., *P. aeruginosa*, *S. aureus*, *S. epidermidis*, *Enterobacter* spp., *Citrobacter* spp., *Serratia* spp., *Aeromonas* spp. and *Acinetobacter* spp., which cause infections in cancer patients predominantly during periods of the neutropenic stage, were, in general, more susceptible to ceftiofime, cefepime, imipenem and WIN-49375 than to the other older agents [92, 93].

The beta-lactamase stability of ceftiofime has been extensively studied [85, 91, 94–96]. Ceftiofime was found to be very stable against the hydrolysis by both plasmid- and chromosomally-coded beta-lactamases of diverse origins. It also possesses a low affinity for the *Enterobacter cloacae* beta-lactamase [97].

For the assay of ceftiofime in the clinical microbiology laboratories, the agar-diffusion test with 30 μ g disks was proposed. The criteria for susceptibility tests with this disk (inhibition zone diameters in mm) and for the dilution assay (μ g/ml growth inhibition) possess breakpoints as follows: susceptible ≥ 18 mm zone (< 8.0 μ g/ml) and resistant ≤ 14 mm zone (≥ 32 μ g/ml) [66].

From the papers referenced throughout this review, it seems prudent to summarize the effects of inoculum and pH on the MIC's, the relationships between MIC and MBC, and the mode of action of ceftiofime.

The inoculum effects on the observed MIC's of ceftiofime have been studied with *P. aeruginosa*, *S. faecalis* and beta-lactamase-producing *S. aureus* strains. No significant differences in MIC values were observed with inocula between 10^4 and 10^6 . However, with a heavy inoculum of 10^7 , the MIC's, especially with some *S. faecalis* and *S. aureus* strains, were found to be four or more dilution steps higher. The effect of pH (ranging between 5.5 and 8.0) of the medium was minimal on the observed MIC values. Nevertheless, above 7.2, the activity was slightly better (as displayed by lower MIC values). This observation is similar to the case with cephaloridine and ceftazidime probably attributable to the pyridine substituent at the 3-position.

With regard to the relationship between MIC and MBC, it was observed that the MBC was the same as the MIC for a large number of the strains tested (about 80%). Nineteen percent of the strains tested displayed a MBC that was one two-fold dilution step higher than that of the MIC of ceftiofime.

Studies using *E. coli* K12-derived penicillin-binding-proteins (PBP) have demonstrated that ceftiofime binds mainly to PBP3 with consequent filamentation and rapid lysis of the bacterial cells. This bacteriolytic property of ceftiofime can explain the excellent in vitro action of the drug. In this respect, ceftiofime is similar to its close chemical relative, ceftazidime. Excellent penetration into bacterial cells, as mentioned above, also contributes to its mode of action.

The chemotherapeutic properties of ceftiofime have been investigated in experimental animals and the drug has been found to possess very good protective effects in the experimental chemotherapeutic mouse model. The data

Table III

Comparative efficacy of cefpirome, ceftazidime and cefotaxime against systemic infections in mice ED_{50} values in mg/kg; modified from reference [98]

Organism	Cefpirome	Ceftazidime	Cefotaxime
<i>S. aureus</i>	0.80	17.68	6.38
<i>S. pyogenes</i>	0.09	1.24	0.21
<i>S. faecalis</i>	71.30	>200.00	>800.00
<i>S. pneumoniae</i>	0.10	2.55	0.40
<i>E. coli</i>	0.03	0.21	0.09
<i>S. typhi-murium</i>	0.07	0.31	0.06
<i>K. pneumoniae</i>	0.06	0.21	0.14
<i>E. cloacae</i>	0.10	0.42	4.05
<i>S. marcescens</i>	2.20	5.20	84.50
<i>P. mirabilis</i>	14.95	30.45	8.44
<i>P. aeruginosa</i>	150.80	72.58	1542.00
<i>P. aeruginosa</i>	21.80	10.48	153.60

presented in Table III, as computed from reference [98], display its excellent ED_{50} values against most infections in comparison with ceftazidime and cefotaxime. In *S. faecalis* infection only cefpirome has a definite ED_{50} . However, in *P. aeruginosa* infections, it is only half as effective as ceftazidime, but significantly more protective than the other anti-pseudomonal cephalosporins in the study [98].

In experimental mouse pneumonia caused by a suspension of a *K. pneumoniae* strain using a nebulizer for infection, cefpirome displayed a more marked bactericidal effect than the other cephalosporins tested (cefotaxime, cefodizime, ceftazidime, cefoperazone and moxalactam). Only cefodizime had the same ED_{50} values (1.1 to 59.1 mg/kg) as cefpirome, the other test compounds were shown to be two to ten times less effective [99].

The pharmacokinetic behaviour of cefpirome has been studied in five animal species (including mouse, rat, rabbit dog and monkey) after a 10 mg/kg subcutaneous, intravenous or intramuscular administration. The peak serum levels obtained after intramuscular administration were 8, 9, 15 and 12 $\mu\text{g/ml}$ in mice, rats, dogs and monkeys, respectively. The elimination half-life displays species variation. It has been shown to be 20 min in mice and 89 min in rabbits (i. v.) but the elimination in rodents is slow (6 h). Cefpirome penetrates well into tissues and body fluids reaching adequate concentrations. It is eliminated mainly by the kidneys with a urinary recovery rate of 15–85% (species and route of administration are responsible for the variations seen in this rate). Biliary excretion is very low for cefpirome (only rat and rabbit data are available). Its serum protein binding profile is very low, 4% in rabbits and 15% in mice [100]. The pharmacokinetic profile of cefpirome is very similar to that of its chemical relative, ceftazidime.

On the basis of the pre-clinical data described above, cefpirome can be considered to be an improved ceftazidime. Since ceftazidime is often referred to as a fourth-generation cephalosporin, cefpirome even more deserves this classification, especially if its activity in *P. aeruginosa* infections proves to be clinically useful. Human trials with cefpirome are currently in progress as communicated by the manufacturer (Hoechst).

Cefepime

Cefepime (BMY-28142) is a recent member of the 3-quaternary ammonium semi-synthetic cephalosporins. It is an aminothiazol methoxyimino cephalosporin containing a quaternized *N*-methyl-pyrrolidine group attached to the 3-position of the methylene cephem (Fig. 2). It is generally available as a sulphate salt.

Cefepime is very active against staphylococci, as well as *P. aeruginosa* ("balanced" antibacterial spectrum) and probably more effective against most members of the *Enterobacteriaceae* than other broad-spectrum cephalosporins, including strains producing beta-lactamase and resistant to other third-generation aminothiazol cephalosporins. It is virtually completely resistant to the common beta-lactamases and possesses a low affinity for these enzymes. All of these characteristics make it a potential candidate for an excellent drug.

The in vitro antimicrobial activity of cefepime used alone [101–108] and in direct comparison with other beta-lactam antibiotics [93, 109, 110] has been investigated in many independent studies. Although various methods and bacterial strains were employed in these studies, the results have been in good agreement. Studies in which ceftazidime and/or cefpirome have been used as control compounds have provided especially useful data. Cefepime has been found to possess a broad, balanced antibacterial spectrum making it the most active cephalosporin against the overwhelming majority of Gram-positive and Gram-negative aerobic and facultative anaerobic bacteria. The in vitro results are briefly summarized below in the order of decreased susceptibility (MIC₉₀ values in µg/ml given in parentheses): *N. meningitidis* (0.008), *H. influenzae* (0.06), *N. gonorrhoeae* (0.02), *M. morgani* (0.05), *P. mirabilis* (0.05), *Salmonella* spp. (0.07), *Shigella* spp. (0.04), *Streptococcus agalactiae* (0.07), *S. pyogenes* (0.03), *S. pneumoniae* (0.05), *E. coli* (0.25), *E. aerogenes*, *E. cloacae*, *E. agglomerans* (0.2–0.4), *P. vulgaris* (0.25), *Providencia* spp. (0.25), *Citrobacter* spp. (0.25), *K. pneumoniae*, *K. oxytoca* (0.3–0.5), *S. marcescens* (2.0), *Clostridium perfringens* (0.1), *S. epidermidis* (0.5–0.8) and beta-lactamase-producing strains of *S. aureus* (1.9–3.8). Against the members of the *Enterobacteriaceae* family, cefepime was found to be the most active of the third- (or fourth)-generation cephalosporins (MIC₉₀ = 1 µg/ml).

Cefepime has been shown to be about half as active (in $\mu\text{g/ml}$) against *P. aeruginosa* (7.0) and *P. cepacia* (5.0) as ceftazidime. It was shown to be weakly active against *P. maltophilia* (21.0), *Acinetobacter* spp. (27.0), *Mycobacterium avium-intracellulare* (16.0), *Listeria monocytogenes* (50.0), *S. faecalis* (64.0), methicillin-resistant *S. aureus* (8.0–32.0) and not active against *B. fragilis*.

Cefepime's MIC values (in $\mu\text{g/ml}$) against the standard quality control strains were found as follows: *E. coli* ATCC 25922 (≤ 0.06), *P. aeruginosa* ATCC 27853 (2.0–4.0), *S. faecalis* ATCC 29212 (≥ 32.0), *S. aureus* ATCC 25923 (1.0–2.0) and *S. aureus* ATCC 29213 (2.0–4.0). These strains should be used regularly in the clinical laboratory when susceptibility tests are performed [103].

In vitro studies have demonstrated that the combination of cefepime with tobramycin often acted in a synergistic manner, while combinations of cefepime with imipenem or polymyxin B usually acted in an antagonistic manner when tested against strains of pathogenic organisms isolated from paediatric cystic fibrosis patients [108]. The combination of cefepime with amikacin has been usually found to be synergistic as well [111].

On the basis of the available in vitro MIC data, which is sometimes overlapping and sometimes conflicting, a rank order reflecting the relative activities of third- and fourth-generation compounds against important bacterial strains can be drawn for comparative purposes. The following comparison has been adapted and updated from reference [62]. The rank order for *Enterobacteriaceae* is cefepime > cefpirome > moxalactam and ceftazidime > cefotaxime > cefoperazone. The relative activities against *P. aeruginosa* are ceftazidime > cefepime > cefpirome > moxalactam and cefotaxime. For *S. aureus* the rank order is cefotaxime > cefepime and cefpirome > cefoperazone > ceftazidime > moxalactam.

The effects of inoculum size, medium composition and pH on the in vitro activity, as well as serum protein-binding and mode of action of cefepime have been extensively studied [101, 112]. The effect of inoculum size on the activity of cefepime was shown to be minimal between inoculum levels of 10^4 and 10^6 . The increase in MIC was 0 to 4-fold, depending upon the bacterial strains tested, but was more pronounced when inoculum levels were above 10^6 colony forming units. The effect of medium composition and pH was negligible over a range of pH between 6.4 and 8.0.

The bactericidal concentration of cefepime has been found to be identical, or very close, to the MIC values for most bacterial strains studied. The primary binding site for cefepime in *E. coli* has been found to be penicillin-binding-protein 3 (PBP3) and in *P. aeruginosa* PBP1 and PBP3. Both of these binding sites are lethal receptors, with consequent formation of filamentation and rapid lysis of the bacterial cells.

Cefepime has been shown to be highly resistant to hydrolysis by many chromosomal and plasmid-mediated beta-lactamases, regardless of whether

they are purified enzymes or bacterial strains known to produce the relevant beta-lactamases. In this respect, cefepime resembles most of the third-generation, and some second-generation, cephalosporins. As it turns out, however, resistance to hydrolytic inactivation by cephalosporinases of Gram-negative bacteria is not always sufficient to ensure therapeutic effectiveness, since in the case of inducible chromosomally-mediated cephalosporinases, resistance during therapy develops with relapse and therapeutic failure. The reason for this is a phenomenon known as "non-hydrolytic barrier", also referred to as "antibiotic trapping" or "sponge effect". In this instance, there is a formation of a long-lived inactive cephalosporin-enzyme complex within the periplasmic space which does not allow the drug to attach to the lethal PBP receptors. This binding to, rather than hydrolysis by, the beta-lactamases is thought to be the major reason and an efficient mechanism of resistance during therapy [64, 113].

Table IV

Interaction of three oxyimino aminothiazol 3-quaternary cephalosporins with chromosomal beta-lactamases of P. aeruginosa and E. cloacae From reference [114]

Cephalosporin	Relative rate of hydrolysis*	Inhibitor activity**
Ceftazidime	<0.001	90
Cefpirome	0.001	70
Cefepime	0.003	30

* Hydrolysis of cephalothin = 100

** Shown as percentage of cephalothin hydrolysis that was inhibited by equal molar concentrations of the test cephalosporin

In this respect, studies have shown that cefepime is unique in that it has been demonstrated to possess the lowest substrate affinity for beta-lactamases [65, 114–116]. Table IV, taken from reference [114], shows that all three oxyiminoaminothiazol 3-quaternary cephalosporins are not hydrolyzed by the chromosomal beta-lactamases (that is they are all almost equally poor substrates) of *P. aeruginosa* and *E. cloacae* (first column in Table IV). Figures in the second column of the table demonstrate that ceftazidime is the best inhibitor of cephalothin hydrolysis (because it is mostly bound to the beta-lactamases in the periplasmic space) and the least efficient inhibitor is cefepime (because it is not bound by the enzymes); cefpirome is in between. Figures in Table V corroborate the supposition that ceftazidime is the most affected by the non-hydrolytic barrier and that cefepime is the least affected as expressed by the difference between MIC values obtained for wild-type cells (little or no enzyme production) and those obtained for the mutant cells which produce abundant enzyme. As expected, cefepime either was not or only about four-

fold less active against the mutants than against the wild-type, while cefpirome was 8 to 16-fold less active, and ceftazidime about 32 to 256-fold less active. These results are the consequences of the relative affinity of the enzyme protein to the drugs. Cefepime has a low affinity (non-complexing) and ceftazidime a higher affinity (complexing) to the beta-lactamases. This is the basis of the non-hydrolytic barrier, another mechanism of bacterial resistance. Cefepime remains very active in most instances since it resists both hydrolytic and non-hydrolytic types of enzymatic inactivation.

Table V

In vitro activity of three oxyimino aminothiazol 3-quaternary cephalosporins against Gram-negative bacilli possessing inducible beta-lactamases and their mutants stably derepressed for beta-lactamase production From reference [114]

Organism	Cephalosporin	MIC in $\mu\text{g/ml}$ for	
		wild type	mutant
<i>E. cloacae</i>	Cefepime	0.06	0.12
	Cefpirome	0.06	0.50
	Ceftazidime	0.06	32.00
<i>E. aerogenes</i>	Cefepime	0.06	0.50
	Cefpirome	0.06	4.00
	Ceftazidime	0.25	64.00
<i>C. freundii</i>	Cefepime	0.06	0.06
	Cefpirome	0.25	2.00
	Ceftazidime	0.50	128.00
<i>M. morgani</i>	Cefepime	0.06	0.06
	Cefpirome	0.06	0.25
	Ceftazidime	0.06	8.00
<i>Serratia</i> spp.	Cefepime	0.06	0.50
	Cefpirome	0.12	1.00
	Ceftazidime	0.25	32.00
<i>P. aeruginosa</i>	Cefepime	16.00	32.00
	Cefpirome	16.00	128.00
	Ceftazidime	4.00	128.00

For the determination of cefepime levels in plasma and urine, a quick and accurate high-pressure liquid chromatography procedure has been developed instead of the usual time-requiring microbiological assay [117]. This development has greatly facilitated pharmacokinetic studies in both animals and humans [118].

The pharmacokinetic properties of cefepime have been determined in mice, rats, dogs and monkeys. For these studies, cefepime was used as either the sulphate salt or the lyophilized dipolar ion reconstituted with sterile water to yield 250 or 500 $\mu\text{g/ml}$ solutions [112, 119].

The pharmacokinetic parameters of cefepime in mice have been shown to be similar to those of ceftazidime. After a 20 mg/kg intramuscular injection, the peak serum concentration was found to be 20.5 mg/kg, the elimination half-life ($T_{1/2}$) was sixteen min and the urine recovery rate about 73%. Rats tolerated the intravenous infusion better than a bolus injection. The parameters were found to be similar to those in mice and were shown to be dose-related. In dogs, after a 10 mg/kg i. v. injection, the $T_{1/2}$ was found to be 1.1 h, there was good tissue distribution of the drug and excretion via the kidneys. Urinary recovery in 24 h was 100% of the unchanged compound. In cynomolgus monkeys, after 10, 100, 300 and 600 mg/kg i. v. infusions, the $T_{1/2}$ was 1.6–1.7 h and all parameters were related in a linear fashion to the dose. Inter-subject variability was found to be low. These results obtained were close to those expected for a dipolar ionic cephalosporin with good distribution within the body. Binding to human serum proteins of cefepime is low, about 20%, and similar to that of ceftazidime [112].

In experimental systemic infections in mice, the protective (therapeutic) efficacy of cefepime against eleven species of bacteria was found to be excellent. For the strains used, the ED_{50} (PD_{50}) values were consistent with the MIC values. With the exception of *P. aeruginosa* infections for which ceftazidime was shown to be better with lower PD_{50} values, cefepime was equally protective, or more, than control cephalosporins such as ceftazidime, cefotaxime, moxalactam or cefoperazone [112].

In experimental pneumococcal meningitis in rabbits, cefepime was found to be as effective as ceftazidime. It penetrated the inflamed meninges and reached dose-dependent bactericidal concentrations in the cerebrospinal fluid [120]. It was also effective in experimental pseudomonal endocarditis, as well as in urinary and respiratory tract, and thigh muscle infections in rats. In another experimental bacteraemia and meningitis model in newborn rats with *E. coli* and group B streptococci, cefepime produced rates of bacterial clearance from the blood and cerebrospinal fluid similar to those of penicillin G or cefotaxime. In effect, cefepime reached higher bactericidal levels (15% of the serum level) than the other two antibiotics [121].

The laboratory and pre-clinical studies with cefepime have been so promising that clinical trials are warranted and are in progress.

DN-9550

DN-9550 is a newer member of the 3-quaternary cephalosporins. Like ceftazidime, it contains a pyridinium ring attached to the 3-methylene group of the cephem nucleus and an imidazolmethoxyimino substituent at the α -position of the aminothiazolacetyl moiety (Fig. 2). The comparative structural

relationships of this compound to other members of the group are presented in reference [35].

The broad in vitro antibacterial activity of DN-9550 is comparable to that of ceftazidime against most species of the family of *Enterobacteriaceae* with some distinct differences [122].

DN-9550 possesses in vitro activity against both Gram-positive and Gram-negative aerobic bacteria. The MIC₉₀ values have been found to be as follows: for *S. aureus* 1.56 µg/ml, *S. epidermidis* 6.25 µg/ml, *S. pneumoniae* and *S. pyogenes* ≤ 0.05 µg/ml, *E. coli* 0.19 µg/ml, *K. pneumoniae*, *K. oxytoca* and *P. mirabilis* 0.39 µg/ml, indole-producing *Proteus* spp. 1.56 µg/ml, *H. influenzae* and *N. gonorrhoeae* 0.78 µg/ml. It showed less activity against *C. freundii*, *Acinetobacter* spp., *P. maltophilia*, *Alcaligenes faecalis* and *B. fragilis*. The main feature of DN-9550 is its high level of activity against both staphylococci and *P. aeruginosa*.

DN-9550 possesses high affinities for PBP1B and PBP3 of *E. coli* and *P. aeruginosa*. This is reflected in its bactericidal activity; the MBC values in µg/ml are the same as the MIC values (in µg/ml) for *E. coli*, *S. marcescens* and *S. aureus*, and twice as high for *P. aeruginosa*.

DN-9550 is resistant to hydrolysis by beta-lactamases of diverse origins (*E. coli*, *S. marcescens*, *P. aeruginosa* and *P. vulgaris*).

DN-9550 was found to be protective in systemic infections of mice caused by *S. aureus*, *S. pyogenes*, *E. coli*, *K. pneumoniae* and *S. marcescens* with ED₅₀ values of 0.6 mg/kg to 4.6 mg/kg, depending upon the species. For *P. aeruginosa*, the ED₅₀ was found to be 10.3 mg/kg.

As for its pharmacokinetic parameters, the i. v. administered DN-9550 (20 mg/kg) to rats and monkeys was distributed rapidly to all tissues, such as kidney, trachea, lungs, liver and muscles. Blood levels of 83.8 µg/ml were found five min after injection. Serum protein binding was found to be 26% in rats and 16% in monkeys.

DN-9550 is excreted via the urine as an unchanged, non-metabolized drug at 97% and 71% of the original dose over a 24 h period in rats and monkeys, respectively.

The broad spectrum and favourable pharmacokinetics of DN-9550 warrant its further evaluation in humans and clinical trials be initiated.

DQ-2556

DQ-2556 is a new, broad-spectrum methoxyimino aminothiazol compound with a basic ring, an oxazole-substituted pyridine group at the 3-position of the cephalosporin structure. It possesses the biological and chemical characteristics of the quaternary ammonium cephalosporins. Its chemical structure is presented in Fig. 2. It is produced as a monosulphate salt.

The in vitro antibacterial activity of DQ-2556 has been compared to those of ceftazidime and cefotaxime [123]. The activity of DQ-2556 was comparable to that of cefotaxime. Against *P. aeruginosa*, it was shown to be somewhat less active than ceftazidime but active against staphylococcal strains. The MIC₉₀ values were found to be as follows: for *S. aureus*, *S. epidermidis* and *S. saprophyticus* 1.50, 3.13 and 1.56 µg/ml, respectively; for both *S. pyogenes* and *S. pneumoniae* 0.10 µg/ml; for *E. coli*, *Klebsiella* spp., *P. mirabilis*, *Providencia rettgeri* and *M. morganii* 0.78 µg/ml or less; for *H. influenzae* and *N. gonorrhoeae* 0.1 µg/ml; and for *P. aeruginosa* strains 6.25–25 µg/ml. *S. marcescens*, *C. freundii* and *Enterobacter* spp. were slightly less susceptible to DQ-2556 than were other species of *Enterobacteriaceae*. Activity of DQ-2556 against *B. fragilis*, *P. maltophilia* and methicillin-resistant staphylococci was poor.

Inoculum effect on the activity of the compound was determined to be species and strain dependent, and ranged from the same to about four-fold higher in the MIC values. The effect of pH on the activity of DQ-2556 was negligible.

The bactericidal concentrations of DQ-2556 were also shown to be strain dependent and for members of the *Enterobacteriaceae* were generally the same as the MIC values or about four times higher at most. Against *P. aeruginosa* DQ-2556 was demonstrated to be less bactericidal than ceftazidime.

Because of its balanced antibacterial activity and other favourable laboratory parameters, DQ-2556 may be a candidate for development, pharmacokinetics, and toxico-pathologic parameters permitting.

BO-1236

BO-1236 is a quite recent member of the quaternary ammonium cephalosporins. It contains the same alpha-substituted aminothiazol side-chain at the 7-position as does ceftazidime and the quaternized *N*-methyl-5,6-dihydroxyisindolinium moiety at the 3-methylene position of the cephem nucleus. Its chemical structure is shown in Fig. 2.

In vitro and in vivo studies have demonstrated that BO-1236 is a potent cephalosporin with good activity against Gram-negative organisms, particularly against *P. aeruginosa*, including those strains resistant to ceftazidime [124]. The in vitro antibacterial activity of BO-1236 is reflected in its MIC₉₀ values as follows: *P. aeruginosa*, *S. pneumoniae* and *S. pyogenes* 0.78 µg/ml; *E. aerogenes*, *E. cloacae*, *C. freundii* and *M. morganii* 1.56 µg/ml (the ceftazidime-resistant strains of these species at 12.5 µg/ml); *H. influenzae*, *Branhamella catarrhalis* 0.78 and 0.39 µg/ml; *S. marcescens* 7.33 µg/ml; *K. pneumoniae* 0.20 µg/ml; *A. calcoaceticus* 0.39 µg/ml; *P. aeruginosa* 0.2 µg/ml with ceftazidime-resistant strains of this organism at 0.78 µg/ml and the gentamicin-resistant strains at 1.56 µg/ml. BO-1236 has been shown to be less active against *S.*

aureus and *S. epidermidis* with MIC₉₀ values of 50 µg/ml. It does not possess any activity against *S. faecalis* or *B. fragilis*. The MBC values have been found to be close to the MIC values, but are influenced by inoculum size.

BO-1236 has been shown to be stable to almost all plasmid- or chromosomally-mediated beta-lactamases with the exception of type I oxyimino-cephalosporin-hydrolyzing enzyme. The resistance to most beta-lactamases is reflected in its excellent activity in vitro against ceftazidime-resistant strains of *S. marcescens*, *E. cloacae*, *E. aerogenes* and *C. freundii* with geometric mean MIC's at or below 1 µg/ml.

In experimental chemotherapeutic studies with mice, BO-1236 was found to be very effective against infections produced by Gram-negative bacteria. The ED₅₀ values for such studies were found to be as follows: *E. coli* 0.05 mg/kg; *K. pneumoniae* 0.04 mg/kg; *S. marcescens* 0.7 mg/kg; and *P. aeruginosa* 1.4–3.9 mg/kg. These values are better than those obtained for ceftazidime or cefotaxime. BO-1236 was found to be less protective in infections caused by *S. aureus* (Smith) where the ED₅₀ was found to be 24.2 mg/kg.

BO-1236 has been shown to be non-toxic in mice. In acute toxicity tests, the LD₅₀ dose was 2201 mg/kg (2081–2329 mg/kg). The maximal tolerated dose (which caused no death) was determined to be 1715 mg/kg. This low toxicity and excellent activity against Gram-negative organisms, especially its antipseudomonal activity, warrant further evaluation of this promising new quaternary cephalosporin.

ICI-194008 and ICI-193428

ICI-194008 and ICI-193428 are newcomers to the group of cephalosporins containing quaternary ammonium substituents at the 3-position. They retain the same side-chain as ceftazidime at the 7-position and possess comparable activity against *P. aeruginosa* but better activity against *P. maltophilia* than does ceftazidime [125]. Their chemical structures are presented in Fig. 2.

In vitro the two compounds displayed relatively comparable activities against the bacterial strains tested. Occasionally ICI-193428 was more active than ICI-194008, but never more than one tube dilution in any assay.

Against members of the *Enterobacteriaceae* family, both compounds exhibited activities comparable to that of ceftazidime but less than that of cefpirome. They were, however, more active than ceftazidime against strains of *E. cloacae*, *E. aerogenes* and *C. freundii* with MIC₉₀ values of 4 µg/ml or less; the same values for ceftazidime were 16–64 µg/ml. Against *P. aeruginosa*, both ICI compounds and ceftazidime have equivalent activity (MIC₉₀ ≤ 8 µg/ml). Against *P. maltophilia*, the ICI compounds were most active with MIC₉₀

values of ≤ 16 $\mu\text{g/ml}$, compared to those of ceftazidime and cefpirome of ≥ 128 $\mu\text{g/ml}$. Against *S. aureus* and *S. epidermidis*, both compounds possess inferior activity (4–16 $\mu\text{g/ml}$) similar to that of ceftazidime. No activity against *Listeria monocytogenes*, *S. faecalis*, penicillin-resistant pneumococci and methicillin-resistant staphylococci could be demonstrated by either compound.

Both compounds appear to be stable to most beta-lactamases and no inoculum effects on the activities were found. In Mueller-Hinton medium, ICI-193428 was more stable than ICI-194008 which lost 20% of its activity during a 24 h period at 37 °C. Both compounds possess favourable biological properties justifying their further evaluation and eventual development.

BO-1232, CM-40874, GR-32620 and an unnamed isoquinoline

The quaternary cephalosporins listed above are in the very early phases of evaluation and are only partially characterized. Discussions on their chemical compositions and brief descriptions can be found in several review papers [31, 63, 67, 126]. Their chemical structures are depicted in Fig. 2. These compounds possess some noteworthy chemical properties and offer some interesting biological properties including anti-pseudomonal activity.

BO-1232 is claimed to possess activity against *P. aeruginosa* ten-times more potent than that of ceftazidime. It is also said to exhibit a high degree of activity against other pseudomonal species. As can be seen in Fig. 2, it contains an ethoxyimino group at the alpha-acetyl position. Its methoxyimino equivalent compound possesses similar activities.

CM-40874 is an S-oxide cephalosporin (Fig. 2) and is a compound which is very active against Gram-negative bacteria including *Pseudomonas* spp. It has, however, no activity against staphylococci. It is extremely stable to hydrolysis by cephalosporinase even at very high concentrations of the enzyme. This is probably due to the fact that it binds to the PBP2 (through its sulphoxide function) in Gram-negative bacilli in a manner similar to amdinocillin (mecillinam), imipenem and aztreonam. It is listed, wrongly or rightly, as a fourth-generation cephalosporin [67].

GR-32620 is a recently synthesized cephem which contains a cyclopropylmethoxyimino group at the alpha-7-acetyl position (Fig. 2). It was included in a comparative in vitro and in vivo evaluation, the results of which are presented in Table VI and Table VII, both taken and modified from reference [62]. It can be seen in these tables that GR-32620 has an antibacterial spectrum similar to that of ceftazidime against Gram-negative bacteria, but possesses a significantly greater activity and efficacy against *S. aureus*. In the in vitro study (Table VI), cephaloridine was added to the quaternary compounds under study for comparative purposes.

Table VI

Comparative *in vitro* activities (MIC's in $\mu\text{g/ml}$) of four quaternary cephalosporins and cephaloridine
Modified from reference [62]

Cephalosporin	Pen. res. <i>S. aureus</i>	Amp. sens. <i>E. coli</i>	Amp. res. <i>E. coli</i>	<i>E. cloacae</i>	<i>H. influenzae</i>	<i>P. aeruginosa</i>
Ceftazidime	8.00	0.10	0.10	0.50	0.06	1.00
Cefpirome	1.00	0.05	0.06	0.06	0.06	4.00
Cefepime	1.00	0.06	0.06	0.06	0.06	4.00
GR-32620	0.20	0.10	0.10	0.50	0.06	2.00
Cephaloridine	0.10	4.00	32.00	> 256	2.00	> 256

Table VII

Comparative *in vivo* efficacy (ED_{50} in mg/kg mouse) of three quaternary cephalosporins and cefotaxime
Modified from reference [62]

Cephalosporin	<i>S. aureus</i>	<i>E. cloacae</i>	<i>K. pneumoniae</i>	<i>Serratia</i> spp.	<i>P. aeruginosa</i>
Ceftazidime	14.0	0.1	0.5	0.1	0.3
Cefpirome	3.6	25.0	0.5	0.2	6.2
GR-32620	0.7	10.9	2.0	1.2	2.7
Cefotaxime	2.1	0.1	0.3	3.9	> 50.0

Isoquinoline is a compound without a code name and/or number and is listed in reference [31] with the remark that it has an antibacterial spectrum similar to that of cefpirome.

In addition to the above compounds published, a large series of quaternary ammonium cephalosporins have been produced, studied and reported at various meetings to possess good bactericidal activities and beta-lactamase stability. The 3-substituents of these compounds consist of a variety of quaternized monocyclic and bicyclic aromatic bases, such as pyridines, pyrrolidines, pyrazines, thienopyridines, furopyridines, cyclopentanopyridines, cyclohexanopyridines, quinolines, isoquinolines and isoindoles. They are zwitterionic polar compounds with high levels of activity against *P. aeruginosa* expected from these betaines.

Some have even displayed interesting pharmacology, namely anticholinergic activity in *in vitro* model tests. The list of these cephalosporins will most likely grow at an exponential rate based upon the rapid emergence of new compounds.

Quaternary ammonium cephalosporins which contain the oxyimino-aminothiadiazol moiety at the 7-position, in place of the somewhat similar oxyiminoaminothiazol substituent, have also been synthesized and studied.

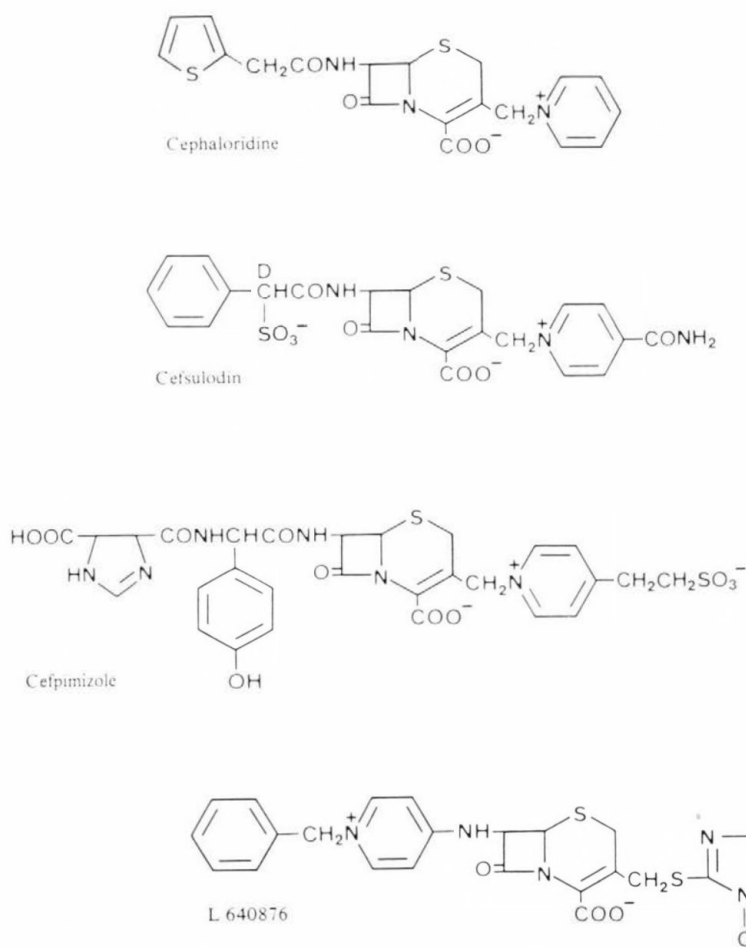


Fig. 3. Chemical structures of three 3-quaternary non-aminothiazol and one 7-quaternary cephalosporins

The reported members of this group are CPG-22495, CPG-22042, EP-APPL 0074645 and FR-17126. They have all reported to exhibit activity against *P. aeruginosa* [63, 126].

It is interesting to note that the introduction of the quaternary heterocyclic (pyridinium) function into the 7-position does not produce the same dramatic antibacterial activity which is observed with the 3-position substitution [31]. One such novel cephalosporin, L-640, 876 (Fig. 3), although, has a wide spectrum of activity against many Gram-positive and Gram-negative bacteria but is still not as potent a compound as those with the 3-position substituted quaternary cephalosporins [127-130].

Non-aminothiazol cephalosporins with 3-quaternary substituents

For the sake of completeness and better appreciation of the significance of the structure-activity relationships as they relate to the 7-substituent aminothiazol-syn-oxyimino and the 3-substituent quaternary ammonium cephalosporins, it seems prudent at this point to deal very briefly with three 3-pyridinium compounds which contain groups other than 7-aminothiazol-acetyl side chains. It can be emphasized at the outset that these compounds are different and somewhat less used or less usable cephalosporins [46, 48, 52]. Two of them, cephaloridine and cefsulodin, are earlier cephalosporins and the third, cefpimizole, is a recent one. Their chemical structures are depicted in Fig. 3.

Cephaloridine

Cephaloridine was the second cephalosporin drug introduced into human therapy in 1964. It possesses a thienylacetamido substituent at the 7-position and not an aminothiazol one (Fig. 3). It has good activity against streptococci, susceptible strains of *S. aureus*, non-beta-lactamase-producing *E. coli* and *K. pneumoniae* but lacks activity against most other members of the *Enterobacteriaceae*. It is sensitive to beta-lactamases and, in addition, it possesses significant renal toxicity, especially at high (over 4 g/day) doses. Because of these shortcomings and despite its advantageous pharmacokinetics (it can be injected i. m. not only i. v.), the clinical usefulness of cephaloridine has become limited [46, 48, 52, 131]. The kidney toxicity of cephaloridine was alarming and attributed to the 3-quaternary pyridine ring. Luckily, this renal toxicity was observed only in this combination of 3- and 7-substituents and was never associated with other 3-pyridinium compounds which contained other 7-position substituents of which the aminothiazol is of paramount importance as previously discussed.

Cefsulodin

Cefsulodin is another substituted 3-pyridinium cephalosporin with an alpha-sulphophenylacetyl group at the 7-position (Fig. 3). It is a narrow-spectrum antibiotic which is highly active against most strains of *P. aeruginosa* with MIC values of 2 µg/ml and 16–32 µg/ml against the beta-lactamase-producing strains. It is also active against *S. aureus* (MIC 2 µg/ml) and other Gram-positive cocci (MIC's 2–4 µg/ml) with the exception of *S. faecalis* which is completely insensitive to cefsulodin. It possesses little or no activity against other Gram-negative bacilli [46, 48, 52, 132, 133]. Cefsulodin is rather resistant to most beta-lactamases [134]. Its antipseudomonal activity, and probably its

beta-lactamase stability, can be attributed to the 3-pyridinium substituent, but the 7-substituent does not confer any more broader antibacterial activity to the compound.

Cefpimizole

Cefpimizole (AC-1370; U-63196E) is a newer member of the substituted 3-pyridinium compounds. It is a betaine internally compensated with the sulphonic acid group. At the 7-position it contains a bulkier carboxyimidazole substituent (Fig. 3) and not an aminothiazol group, this makes a difference. The compound possesses activity against a broad-spectrum of Gram-negative bacteria, especially *P. aeruginosa*. This activity can be attributed to the 3-pyridinium substituent. No significant activity against Gram-positive microorganisms has been described for cefpimizole. Unlike the 7-aminothiazol group, this 7-substituent does not contribute any spectrum-broadening properties. Cefpimizole possesses activity against many ampicillin-resistant strains and has stability against hydrolysis by chromosomal beta-lactamases. The compound has two interesting characteristics, namely the augmentation of phagocytic function of human macrophages and neutrophils and the demonstration of greater in vivo activity than could be expected from its in vitro activity [135–138]. Although it is not a broad-spectrum compound as compared to the oxyimino-aminothiazol quaternary cephalosporins, it is being studied in humans because of its antipseudomonal activity and interesting biological properties [139–142].

These three 3-pyridinium cephalosporins with varied substituents at the 7-position clearly demonstrate their inferior activities when compared to their oxyiminoaminothiazol-containing 3-quaternary counterparts for broadness of antibacterial spectra, magnitude of potency and intensity of beta-lactamase stability.

Although it is not the intention of this review to cover other than the quaternary cephalosporins, in closing, it should be mentioned that quite recently various C-2 quaternary heterocyclic alkylthio carbapenems series have been synthesized and many of them showed excellent wide antibacterial spectra, including *P. aeruginosa*, and greater stability than the starting compound toward the renal dipeptidase, dehydropeptidase I. This finding opens new therapeutic possibilities to the carbapenems [143–145].

Concluding remarks

The fortuitous discovery and introduction of the *syn*-oxyiminoaminothiazol moiety to the 7-acylamino position of the cephem nucleus has increased the in vitro potency and resistance to beta-lactamase hydrolysis of the cephalo-

sporins to levels never before observed. The introduction of pyridinium and other heterocyclic quaternized mono- and bicyclic aromatic bases onto the 3-position of the cephem has led to even more potent and broad-spectrum, including *Pseudomonas* spp., compounds with improved pharmacokinetics and biological properties.

The 3-quaternary ammonium cephalosporins represent a "new class" of the cephalosporin antibiotics with "balanced" antibacterial spectra. They are not only extremely interesting compounds from the chemical, microbiological and pharmacological points of view, but they are very useful drugs from the therapeutic point of view. Some of them are already used in the treatment of bacterial infections with great success, replacing the toxic aminoglycoside antibiotics and several of them are in advanced clinical trials.

It is a fascinating and long way from the observation of the antagonistic property of the *Cephalosporium acremonium* strain in Italy to the scientific insight and isolation of cephalosporin C in England to the industrial ingenuity and improvement in the production by mutant strains to the industrial production of the nucleus, the 7-aminocephalosporanic acid (7ACA) leading to the introduction into therapy of the first cephalosporin drug in the USA. The story of these heroic endeavours has been recounted in an early review [146]. These facts portray the international nature of drug research and development, their large-scale production and marketing for the benefit of patients all over the world.

The new class of 3-quaternary ammonium cephalosporins have already lived up to their expectations as excellent, potent and well-tolerated anti-infective agents. The continuous extensive research effort in this promising class of compounds is destined to produce even more potent, safe and useful cephalosporins and monolactams.

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NOTE ADDED IN PROOF

Since the submission of the manuscript of this paper an additional methoxyimino-aminothiazol quaternary cephalosporin, E1040, has been published [147, 148]. It is presently being tested in the clinic.

[THERAPY OF AIDS PATIENTS—CLASSICAL STRATEGIES AND PROPOSALS FOR NOVEL THERAPEUTIC APPROACHES

(A SHORT REVIEW)

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Introduction	361
Treatment of opportunistic infections and neoplasms	362
Attempts for stimulating, reconstituting or suppressing the immune response in AIDS patients	362
Specific antiretroviral therapy	364
A refined dHIV construct	364
Combination of transfusion of dHIV harbouring leukocytes and passive immunotherapy ..	368

Introduction

Acquired immune deficiency syndrome (AIDS) is a transmissible disease resulting in depletion of the helper-inducer ($T4^+$ or $CD4^+$) T lymphocyte subset [1–3]. Human immunodeficiency virus (HIV, first designated as HTLV-III, LAV, ARV), an exogenous human retrovirus, the primary etiological agent of AIDS [4, 5] replicates in the $T4^+$ lymphocytes [6] and its replication is associated with an impaired cell proliferation and appearance of a pronounced cytopathic effect. Immunosuppressive proteins released after cell lysis and triggering of autoimmune reactions against $T4^+$ lymphocytes also contribute to the complex immunodeficiency observed in AIDS patients [7]. HIV can infect several cell types other than helper-inducer T cells (follicular dendritic cells of the lymph node reticulum, monocytes, macrophages, B cells, EBV transformed lymphoblastoid cell lines, microglial cells [8]). Most of these cells also express the $T4$ molecule in some extent. Viral replication and the consecutive $T4$ cell depletion and dysfunction of other target cells may account for the

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major clinical signs of AIDS: lymphadenopathy, profound immunological abnormalities resulting in opportunistic infections and perhaps in opportunistic tumours such as Kaposi's sarcoma and B cell lymphoma, and neurological diseases [1-3, 8].

As death in most AIDS patients is caused by opportunistic infections (see Table I), the outcome of the disease could probably be improved (i) either by developing more effective antimicrobial treatments, or (ii) by reconstituting the defective immune response [3, 9]. Considering AIDS as a virus-induced autoimmune disease, (iii) immunosuppressive treatment, despite its potential hazards, has also been accomplished [10].

Based on data concerning the replicative cycle of HIV, (iv) specific antiretroviral treatments could also be designed, assuming that progression of the disease can be inhibited or reversed by reducing virus spread [11-14]. We summarize results of treating opportunistic infections and neoplasms occurring in HIV-infected patients and outline the prospects for immunotherapy and antiretroviral therapy. Finally, we would like to delineate potential benefits of new therapeutic approaches.

Treatment of opportunistic infections and neoplasms

The most frequent opportunistic infections and neoplasms of AIDS patients, their clinical manifestations and the drugs used for their therapy (if available) are summarized in Table I. There are frequent recurrences after finishing treatment of these infections and some infections are refractory.

Attempts for stimulating, reconstituting or suppressing the immune response in AIDS patients

Considering the broad range of life-threatening infections occurring in AIDS patients, immunostimulation or immune reconstitution seemed to be a rational therapeutic approach. Table II summarizes the various immunotherapeutic interventions and their consequences. Based on data of Table II, we may conclude that different kinds of immunotherapy proved to be ineffective. Therapy with a combination of several lymphokines was suggested to be potentially more effective [8], though by increasing the number of T-helper cells the number of potential target cells for HIV replication would increase, too. In case of adoptive immunotherapies, the patients have not been effectively reconstituted either in consequence of the absence of a responsive host cell population or in consequence of destruction of the transferred cells as a result of infection by HIV. As to the potential therapeutic use of *in vitro* IL-2 treated

Table I

Treatment of the most frequent opportunistic infections and neoplasms of patients with AIDS
Based on references [1-3, 9, 15, 16]

Organisms	Clinical manifestations	Drugs	Note
A. Infections			
<i>Pneumocystis carinii</i>	Pneumonia	Trimethoprim-sulfamethoxazole and/or pentamidine	High frequency of adverse reactions and recurrences
<i>Toxoplasma gondii</i>	Encephalitis Dissemination	Pyrimethamine and sulfadiazine (Clindamycin?)	Antitoxoplasma therapy must often be supplemented with corticosteroids to reduce cerebral edema
<i>Cryptosporidium</i>	Enterocolitis	(Spiramycin?)	Untreatable with currently available regimens
<i>Candida</i> species	Stomatitis Esophagitis Dissemination	Nystatin or ketoconazole Amphotericin B or ketoconazole Amphotericin B	Recurrences in a high proportion of patients
<i>Cryptococcus neoformans</i>	Meningitis	Amphotericin B and flucytosine	Nephrotoxicity; Bone marrow depression. Recurrences
<i>Mycobacterium avium-intracellulare</i>	Dissemination	—	Untreatable with currently available regimens
<i>Mycobacterium tuberculosis</i>	Dissemination	INH and Rifampin and Ethambutal	
Cytomegalovirus	Retinochorioiditis Dissemination	— Hyperimmune gamma globulin(?)	Untreatable with currently available regimens
Herpes simplex	Mucocutaneous lesions	Acyclovir	Recurrences
Herpes zoster	Localized skin lesions Dissemination	Acyclovir	Recurrences
B. Neoplasms			
Kaposi's sarcoma		Viblastine VP.16 (epidophyllotoxin) Alpha interferon	No major impact on underlying immune deficiency
Burkitt-like lymphoma Primary central nervous system lymphoma non-Hodgkin's lymphoma Squamous cell carcinoma of the oral cavity, oesophagus and rectum			We are not aware of any efficient treatment

autologous CD8⁺ cells [21], such cells may suppress HIV replication, but would not be expected to replace the functions of depleted T4⁺ cells. Concerning immunosuppressive treatment, inhibition of T cell stimulation (and thereby viral replication) and autoimmune cytotoxic mechanisms may temporarily improve the T4⁺ lymphocyte count, but may lead to serious clinical consequences (for example fulminant *Pneumocystis carinii* pneumonia), too [10].

Specific antiretroviral therapy

The known steps in the replicative cycle of HIV are all potential targets for antiviral therapy (Table III). It should be noted that inhibition of virus absorption or release may not stop cell to cell spreading of HIV [38]. As to the reverse transcriptase inhibitors, in vitro inhibition of HIV replication does not parallel with clinical benefit. AZT, the most promising drug and the other inhibitors have considerable toxicity, and we are not aware of any reported healings following AZT therapy. It should be also considered that inhibition of reverse transcriptase activity has no influence on integrated HIV proviruses, which can be activated after stopping of treatment.

Complementary (anti-sense) oligodeoxynucleotides capable to form base pairs with important regions of HIV genes and mRNAs were also suggested for use in AIDS therapy; in this case problems of penetration through the cell membrane and enzymatic degradation should be solved. "Anti-sense" virus constructs would consist of HIV genes with one or more gene(s) in inverted ("anti-sense") orientation. If the *env* gene would be inverted, binding to HIV-specific receptors and as a consequence penetration into the cells would become more difficult; if any of the other genes would be inverted, the cytopathic effect of *env* products should be eliminated.

We suggested earlier that leukocytes harbouring an artificially constructed, defective, noncytopathic HIV (dHIV) provirus (Fig. 1) could be used for the therapy of AIDS patients. dHIV-harbouring leukocytes may reconstitute the patients' immune system, since they are expected to be — at least partly — resistant to superinfection by wild type HIV (wtHIV). dHIV would also slow down the spreading of wtHIV by interfering with its replication and receptor binding. Thus, dHIV harbouring leukocytes and the progeny virions containing dHIV genomes could combine the advantages of immunotherapy and specific antiretroviral therapy.

A refined dHIV construct

In case of our original dHIV construct the basal level of dHIV *env* gene expression is expected to be low in dHIV harbouring cells, as there are deletions in genes coding for transactivating proteins as shown in Fig. 1. High level

Table II*Immunotherapeutic interventions in AIDS*

Based on references [3, 13, 18] if otherwise not specified

Manipulation	Note
A. Administration of lymphokines	
Alpha interferon	Effective in treatment of patients with Kaposi's sarcoma No effect on the underlying immune deficiency
Gamma interferon	Ineffective
Interleukin 2 (IL-2)	Ineffective
Thymic hormones and factors	Ineffective
B. Administration of gamma globulin	Prevents recurrent pyogenic infections (a major problem in pediatric AIDS)
C. Administration of anti-HIV antibodies	Suggested therapeutic approach [23]
D. Plasma exchange	Ineffective
E. Erythrocyte transfusion	Ineffective
F. Administration of immunostimulatory drugs	
Isoprinosine	Ineffective
Azimevon	Ineffective
Cimetidine	Ineffective
Indomethacin	Ineffective
G. Adoptive immunotherapy	
Transfusion of heterologous peripheral lymphocytes	Ineffective
Bone-marrow transplantation (heterologous)	Ineffective [19]
Thymic transplant (heterologous)	Transient effect in some patients
Transfer of CD8 ⁺ lymphocytes treated with IL-2 in vitro (autologous)	Suggested therapeutic approach, CD8 cells inhibit HIV replication in vitro [21]
Transfer of leukocytes harbouring an artificially constructed, defective, non-cytopathic (dHIV)	Suggested therapeutic approach, dHIV may prevent superinfection of transferred cells by wild type HIV, see also table III/I [17]
H. Immunosuppressive therapy	
Cyclosporin A	Danger of fulminant infections [10]
Immunotoxins directed to CD4 cells	Suggested therapeutic approach [20]
Antibodies directed to CD4, class II, HLA or IL-2 receptor molecules	Suggested therapeutic approach [22, 23]
I. Administration of soluble T4 (CD4) protein	Suggested therapeutic approach; soluble T4 protein inhibits HIV replication and syncytium formation in vitro. See references in [24]

dHIV expression and replication could occur only in the presence of wtHIV, which would act as a helper virus. As a consequence of low level of dHIV-encoded *env* proteins, the transfused leukocytes would have only a low level of resistance to superinfection by wtHIV. As effective immune reconstitution is of vital importance in the treatment of AIDS patients, we would like to present here a modified dHIV construct designed to ensure a higher basal level of

Table III*Specific antiretroviral therapies attempted or suggested) in AIDS*

Based on references [11-14] if otherwise not specified

HIV-specific target	Agent	Note
A. Bindings of virions to T4 receptors	Antibodies directed to env proteins of HIV or to T4 receptors	See Table II.
	Soluble T4 (CD4) proteins	See Table II.
	Peptide T (and its analogues)	A threonine rich octapeptide blocking HIV infection of human T-cells in vitro [25]
	HF1.7 anti idiotypic antibody	Mimics CD4 determinants; binds HIV; inhibits HIV infection of human T-cells in vitro [26]
	AL-721 Attenuated HIV strain	Blocks adsorption and/or penetration May infect T-helper cells or stem cells which have escaped infection with virulent HIV and may protect them from virulent HIV infection [34]
B. Reverse transcriptase	AZT (3'-azido-3'-deoxythymidine)	Delays but does not prevent virus production after HIV infection of T-cells in vitro [27]; prolongs survival and delays appearance of opportunistic infections in patients with AIDS or ARC; toxic, induces chromosomal abnormalities [28-30]
	ddC (2'-3'-dideoxycytidine)	Controlled therapeutic trials are in progress
	Suramin	Toxic, no improvement in immunologic function, no substantial suppression of HIV replication, several patients appeared to worsen clinically
	HPA-23	Controlled therapeutic trials are in progress
	Ribavirin (1-B-D-ribofuranosyl-11-1,2-triazole-3-carboxamide)	Unclear mechanism of action. Inhibits development of AIDS in patients with persistent generalized lymphadenopathy
	Phosphonoformate	Acute renal failure developed in the patients treated
C. Transcription provirus	"Anti sense" oligodeoxynucleotides	Oligodeoxynucleotides complementary to HIV genes block expression of the viral genome [31]
	"Anti sense" virus	mRNAs transcribed from inverted viral genes may block transcription of wild type HIV genome [32, 33]
D. Transactivation	Inhibitors of tat and art/trs proteins "Anti sense" oligodeoxynucleotides "Anti sense" virus	Suggested approach [39, 40]
E. Translation of viral mRNAs	"Anti sense" virus	mRNAs transcribed from inverted gene(s) of "anti sense" virus would form base pairs with HIV mRNAs

Table III (continued)

HIV-specific target	Agent	Note
F. Modification and processing of viral proteins	dNM (1-deoxynojirimycin); castanospermine Specific inhibitors of the HIV-1 pol-protease	Glucosidase I inhibitors interfere with HIV-induced syncytium formation and viral infectivity in vitro [35] Suggested therapeutic approach [35]
G. Budding	Interferons (?)	See Table II
H. Unknown target	Missmatched double-stranded RNA Xanthate D609	Protects T-cell lines from HIV infection in vitro by a mechanism distinct from interferon induction [37]. Improves immunological function and controls HIV replication in patients with ARC or lymphadenopathy syndrome [44] Affects the turnover of phosphatidic acid and phosphatidyl-inositol [45], and G. Sauer, personal communication
I. More than one target	dHIV "Anti sense virus" and "Anti sense" oligodeoxynucleotides	Introduction of an artificially constructed, defective, noncytopathic HIV into the patients organism may inhibit replication of wild type HIV as well a binding of the wild type virus to its receptors [17] see also Table II. G See C, D, E

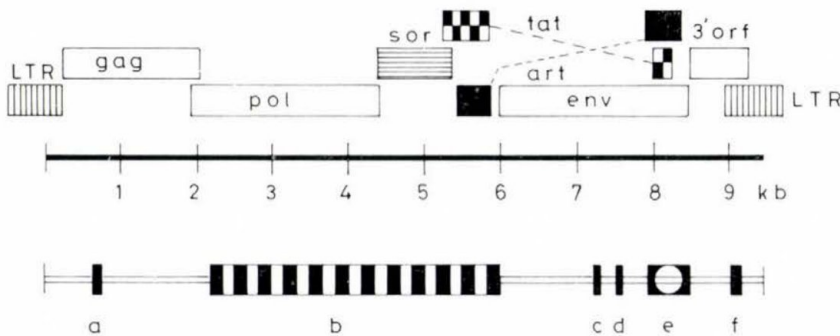


Fig. 1. Proposed characteristics of a refined defective HIV to be constructed. Parallelograms are indicating long terminal repeats (LTR) and genes of gag, pol, sor, tat, art (trs), env and 3' orf of the wtHIV genome [46, 47]. Proposed modifications are shown below the map. Black boxes indicate deletions. Box b indicates deletions (or mutations) in pol, sor and trans-activating genes. Deletions c and d result in impairing wild type characteristics (adsorption and fusion) of HIV. Deletion of the carboxy terminus of env (e) is suggested to be replaced by a sequence coding for a marker polypeptide (white circle) which seems to be necessary to reduce toxic properties of env protein. Deletion f removes the negative regulatory element of LTR and impairs 3' orf (see references in 17). All of the above modifications were proposed for our original dHIV construct [17]. In the refined dHIV construct deletion f is suggested to be replaced by a T-cell specific enhancer-like sequence [41] to ensure a higher basal level of dHIV env expression and superinfection resistance in T-cells. Deletion in the refined dHIV construct eliminates a domain in gag thought to be contributing to immune suppression by HIV [43]

dHIV *env* expression, and therefore a higher level of superinfection resistance in T cells (the most important cell population regarding immune reconstitution of AIDS patients) harbouring dHIV. We propose to insert a putative human T-cell specific enhancer-like sequence [41] into the U3 region of dHIV LTR (Fig. 1). This enhancer-like sequence would replace a negative regulatory element [42] in the vicinity of the TATA box. Since the T-cell specific enhancer-like sequence is not expected to function in other cell types (e. g. in monocytes), wtHIV could infect a certain portion of these non-T cells expressing only low levels of dHIV *env*. The simultaneous presence of dHIV and wtHIV in the cells would result in the generation of progeny virions of mixed phenotype containing either dHIV or wtHIV genomes [17]. This way the efficiency of immune reconstitution would be improved and the advantages of specific suppression of wtHIV-spreading by dHIV would be maintained. A further refinement of the original dHIV construct consists in deletion of a sequence in the *gag* gene coding for an epitope (amino acids 86–115 in p17) immunoreactive with thymosin α_1 [43]. This deletion eliminates an important domain in p17 protein thought to be contributing to the immune suppression by HIV [43].

Combination of transfusion of dHIV harbouring leukocytes and passive immunotherapy

A basic assumption of antiretroviral treatments of AIDS patients is that progression of the disease can be favourably influenced by reducing virus-spread. A complete elimination of the virus, however, seems to be practically impossible, as there are latently infected cells harbouring HIV proviruses and virus variants (having altered biological and immunological properties) in the patients' organism. If transfusion of dHIV harbouring leukocytes would be ineffective, its combination with passive immunotherapy could be attempted. Anti-dHIV *env* antibodies (preferably human monoclonals) or immunotoxins could be used for the elimination of HIV-harbouring cells expressing viral proteins in combination with antibodies directed against wtHIV. This is possible, as dHIV genome-harbouring progeny virions are of mixed phenotype having both dHIV *env* and wtHIV *env* proteins and therefore may superinfect cells harbouring latent wtHIV genomes as well.

To avoid immunosuppressive consequences of such an intervention, it should be accompanied or followed immediately by a novel transfusion of dHIV harbouring leukocytes. This time, however, cells harbouring a dHIV variant coding for an altered dHIV *env* protein (which does not react with the antibody used for passive immunotherapy) would be used.

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We are fully aware of the fact that our proposal could be considered as an extravagance of imagination. But, taken into consideration the unique character of AIDS and the dynamic progress in exploiting the achievements of molecular biology, we do hope that our proposal will not be received as a meaningless fantasy.

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PRODUCTION OF AMYLOLYTIC ENZYMES IN CULTURE BY *BOTRYODIPLODIA THEOBROMAE* AND *SCLEROTIUM ROLFSSII* ASSOCIATED WITH THE CORM ROTS OF *COLOCASIA ESCULENTA*

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Extracellular amylase was detected in culture filtrates of *Botryodiplodia theobromae* and *Sclerotium rolfsii*. During 10 days incubation *S. rolfsii* produced more amylase than *B. theobromae*. *B. theobromae* produced the greatest amount of amylase at 25 °C, while *S. rolfsii* at 30 °C. Both organism exerted the highest amylase activity at pH 6–7. In starch-free medium extracellular amylase was in very low quantities. There was a positive correlation between increase in starch concentration and production of extracellular amylolytic enzymes.

A variety of microorganisms has been found responsible for the storage rots of corms of *Colocasia esculenta* and closely related genera in some countries [1–7]. An anonymous worker [8] in Hawaii reported that the carbohydrate fraction of cocoyam is made up of crude fibre 1.47%, starch 71.91%, reducing sugar 0.52%, sucrose 0.11%, pentosans 2.58% and dextrin 0.52%. Oyenuga [9] reported that cocoyam corms contained starch equivalent of 23.79 g per 100 g food. Coursey [10] also reported that the carbohydrate content of cocoyam was between 13 and 29%.

It has been established by some workers that during the pathogenic process the food contents of the host tissues are greatly reduced. Ogundana et al. [7] investigated the effect of some fungi on the carbohydrate content of some species of *Dioscorea* and reported that there was a reduction in the quantity of carbohydrates, mainly starch and sugars, present in the tubers during pathogenesis.

Arinze et al. [11] reported that the carbohydrate, protein and lipid contents of sweet potatoes decreased remarkably and that the concentration of glucose increased after infection by *Botryodiplodia theobromae*. Nwufu and Fajola [12] also reported that *B. theobromae* and *Sclerotium rolfsii* which were the most important rot pathogens of *Colocasia esculenta* reduced the carbohydrate, protein and lipid contents of cocoyam after infection.

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The major food constituent of the corms of *C. esculenta* is starch and its degradation by any organism depends on its inherent ability to produce amylolytic enzymes. It was on this premise that investigations were conducted on the conditions of amylolytic enzyme production in culture by *B. theobromae* and *S. rolfssii*.

Materials and methods

Test organisms. The test organisms used for the experiments were isolates of *B. theobromae* and *S. rolfssii* obtained from rotten corms of *C. esculenta* during storage in South Eastern Nigeria [6]. The isolates were stored on slants of Potato Dextrose Agar (PDA) in McCartney bottles and were subcultured when needed on plates of PDA.

Medium. The medium used for the production and quantitative estimation of extracellular amylases in culture was devised by Chapman et al. [13]. The organisms were grown on Starch Yeast Extract (SYE): soluble starch, 5.0 g; yeast extract, 2 g; KH_2PO_4 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; distilled water to 1 litre; sterilized at 121 °C for 15 min. The organisms were also grown in a medium designated YE which contained all the above components except soluble starch. The pH of both SYE and YE after autoclaving was 5.4.

For growth measurement one 5 mm disc of fungal colony was used to inoculate each Erlenmeyer flask with 30 ml medium. The flasks were incubated at 30 °C for 10 days. The mycelial mats were filtered from the culture on already weighed Whatman filter paper No.1. The filtered mycelial mats were dried in the oven at 75 °C for 24 h to constant weight.

Assay of enzyme preparation. The amylolytic activity of the culture filtrates was determined by using the method of Chapman et al. [13]. One ml of culture filtrate was mixed with 9 ml of starch solution (1.0% soluble starch in 0.02 M NaH_2PO_4 and 0.006 M NaCl at pH 6.0) and incubated for 1 h at 30 °C. The reducing sugars produced were determined using the dinitrosalicylic acid reagent (DNSA) method. A standard curve was obtained by determining absorbance of 1 ml samples of known aqueous maltose solutions of 0.1, 0.2, 0.4, 0.6, 1.0 and 2.0 mg/ml. The absorbance values read from the standard curve were reported as units of amylase (one unit being the amount of enzyme in 1 ml filtrate which releases 1 mg of reducing sugars from 1% starch solution in 1 h at 30 °C at pH 6.0).

Effects of temperature and pH. Flasks containing the different media for the production of amylase were inoculated with the test fungi and incubated at 5, 10, 15, 20, 25, 30, 35 and 40 °C for 10 days. For the effect of temperature on enzyme activity the enzyme-substrate mixtures were heated at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60 and 70 °C in a water bath for 1 h before amylase activity was determined. The pH of each enzyme assay medium was adjusted to different levels (pH 2–9).

Effects of carbon sources. Sucrose, glucose, starch, carboxymethyl cellulose (CMC), pectin, fructose and maltose were tested in 2% solution of the substrates in the basal medium (30 ml/flask). Incubation lasted for 10 days at 30 °C. The enzyme activities of the culture filtrates were determined. Different concentration of starch on amylase production was also investigated. Thirty ml portions of yeast extract medium with 0–5% starch in 125 ml Erlenmeyer flasks were inoculated with the test fungi and incubated at 30 °C for 10 days.

Results

B. theobromae and *S. rolfssii* produced extracellular amylases in culture at varying degrees. *S. rolfssii* produced more amylase in culture than *B. theobromae* during the 10-day incubation period (Table I). The production of amylolytic enzymes increased with increase of the incubation period. The medium that lacked starch showed little amylase activity which may be due to the yeast extract in the medium.

Table I

Mycelial growth and production of amylase by B. theobromae and S. rolsii on starch yeast extract (SYE) and yeast extract (YE) media during 10 days incubation at 30 °C

Time of incubation (days)	SYE		YE		SYE		YE	
	Mycelial dry wt., mg/30 ml	Amylase units	Mycelial dry wt. mg/30 ml	Amylase units	Mycelial dry wt. mg/30 ml	Amylase units	Mycelial dry wt. mg/30 ml	Amylase units
2	24.8+2.6	0.12+0.01	19.4+1.2	0.00	32.7+1.9	0.08+0.01	11.6+0.9	0
4	54.6+3.4	0.28+0.03	25.6+0.9	0.00	64.8+3.2	0.26+0.05	26.4+2.1	0.02+0.01
6	84.7+3.0	0.56+0.12	49.8+2.4	0.02	89.5+3.4	0.49+0.04	32.3+2.6	0.04+0.01
8	91.4+4.8	0.58+0.13	54.7+2.7	0.04+0.01	108.6+14.6	0.67+0.12	42.7+1.9	0.08+0.01
10	91.6+2.3	0.62+0.21	68.6+1.9	0.04+0.01	118.2+8.7	0.72+0.09	45.4+2.3	0.12+0.01

Data are means of 6 determinations in 2 separate experiments

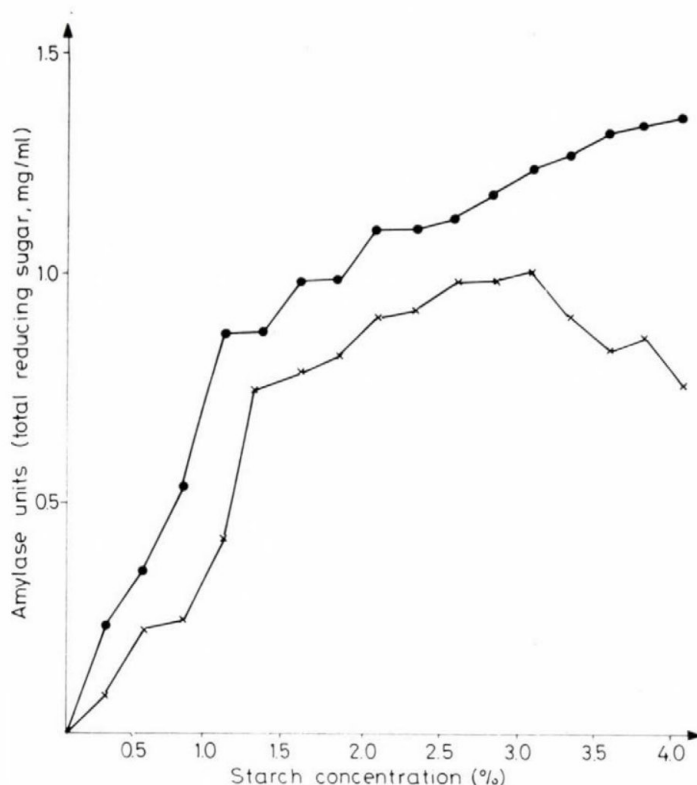


Fig. 1. Effect of starch concentration on the production of amylase by *B. theobromae* (○—○) and *S. rolfsii* (×—×)

The effect of different concentrations of starch on amylase production showed a linear relationship between starch concentration and amylase production up to 3% for *B. theobromae* and even at 5% for *S. rolfsii* (Fig. 1).

The greatest amount of amylase was produced by *B. theobromae* at 25 °C, while by *S. rolfsii* at 30 °C. *B. theobromae* did not produce amylases at 10, 35 and 40 °C, while *S. rolfsii* at 40 °C (Fig. 2).

When the culture filtrate-starch assay medium was incubated at different temperatures, the peak of amylase activity for both organisms was at 50 °C (Fig. 3). Amylase produced by *B. theobromae* did not show any activity at 10 and 70 °C, while amylase produced by *S. rolfsii* was denatured at 70 °C. Amylases produced by the two organisms seem to tolerate high temperature.

The best range for the amylase activity of *B. theobromae* and *S. rolfsii* was pH 6–7 (Fig. 4).

The type of carbon source in the medium invariably affected the amount of amylase produced (Table II). The highest amount of amylase was produced in the medium containing starch, however, amylase production still occurred in

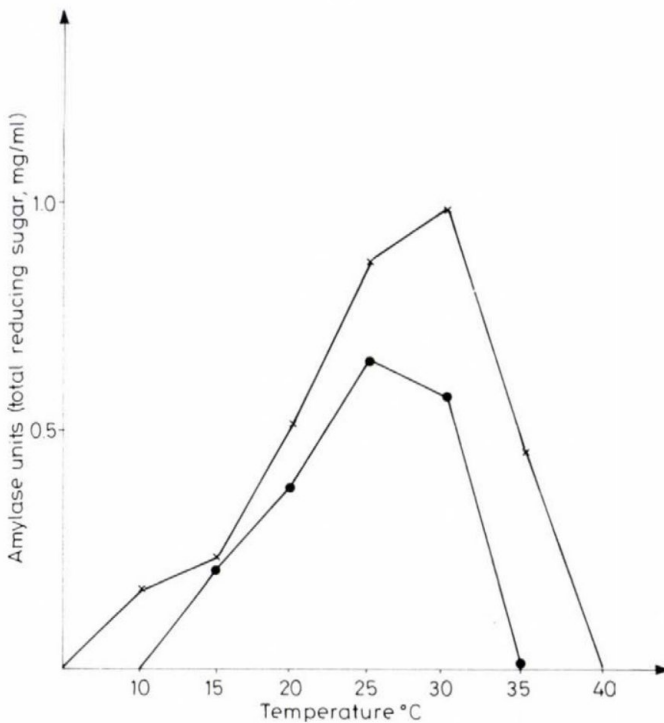


Fig. 2. Effect of temperature on the production of amylases by *B. theobromae* (○—○) and *S. rolfsii* (×—×)

the absence of starch. The two organisms produced little quantity of amylase in glucose, sucrose, CMC, maltose and fructose. The negligible amount of amylase produced in media containing carbon sources other than starch may be due to the yeast extract content of the medium.

Discussion

The two organisms, *B. theobromae* and *S. rolfsii* produced extracellular amylases in culture medium containing starch and other carbon sources but the greatest amount of amylase was produced in medium containing starch as the sole carbon source. However, Adisa [14] reported that *B. theobromae* isolated from rotted citrus fruits did not produce extracellular amylase but exhibited luxuriant growth in a medium containing starch. Corkrane [15] suggested that there was no relationship between utilization of starch for growth and the ability to produce extracellular amylase. Umezurike [16] reported on the inducible nature of the production of amylolytic enzymes of *B. theobromae*. He reported that there was no amylase activity in the enzyme filtrate obtained from culture media containing only glucose, cellobiose or cellulose as the sole

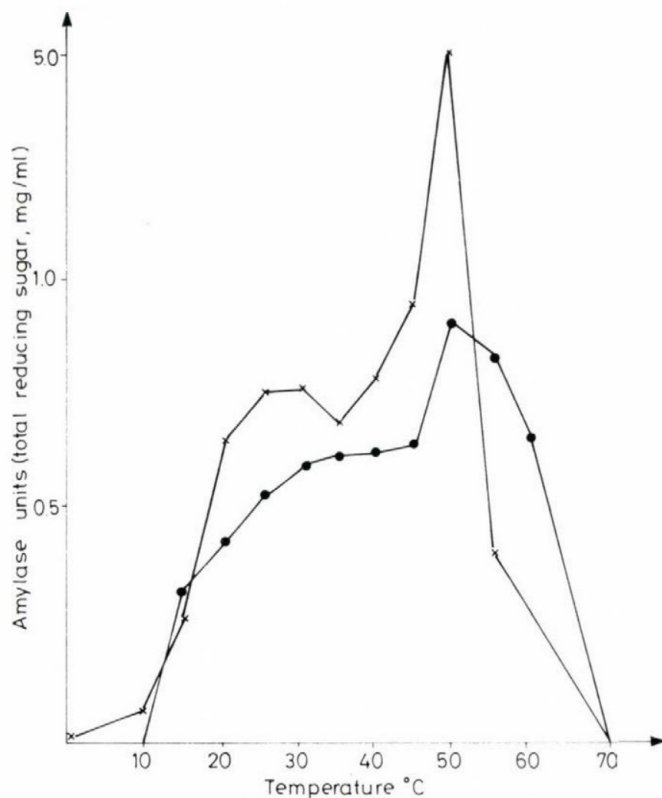


Fig. 3. Effect of temperature on the activity of amylases produced by *B. theobromae* (○—○) and *S. rolfsii* (×—×)

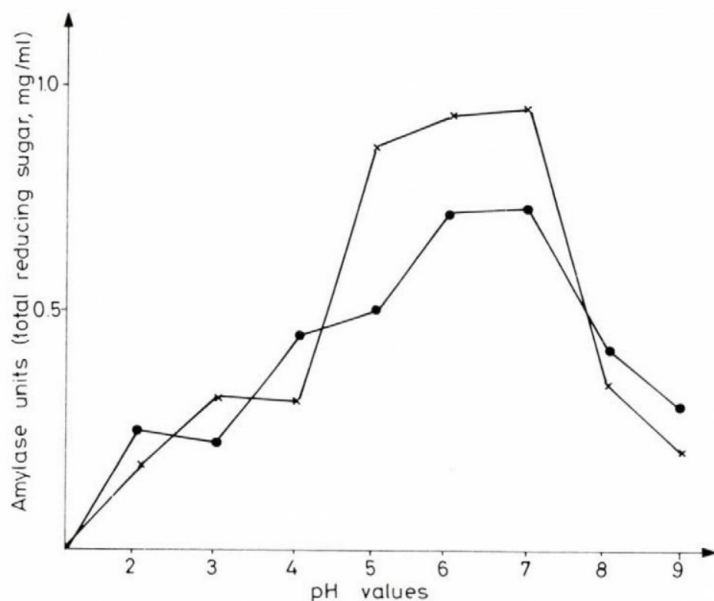


Fig. 4. Effect of pH on the activity of amylase produced by *B. theobromae* (○—○) and *S. rolfsii* (×—×)

Table II

Mycelial growth and production of amylase by B. theobromae and S. rolsii in media containing different carbon sources incubated for 10 days at 30 °C

Carbon source	<i>B. theobromae</i>		<i>S. rolsii</i>	
	Mycelial dry wt. mg/30 ml	Amylase units	Mycelial dry wt. mg/30 ml	Amylase units
Glucose	109.6+5.4	0.06+0.01	100.2+3.4	0.14+0.04
Sucrose	114.4+5.2	0.04+0.01	102.0+1.9	0.11+0.02
Starch	91.6+3.8	0.62+0.12	108.6+5.4	0.72+0.12
CMC	76.4+4.2	0	64.5+5.2	0.09+0.01
Pectin	81.6+3.4	0	72.8+3.5	0.13+0.02
Maltose	91.6+3.4	0.02+0.01	104.7+2.4	0.13+0.01
Fructose	88.4+2.3	0.04+0.01	101.7+3.6	0.14+0.02
Control	60.6+1.8	0.04+0.02	46.4+2.1	0.12+0.03

CMC = Carboxymethyl cellulose

Data are means of 6 replicates in 2 separate experiments

carbon sources but detected appreciable amylase activity in a medium containing starch. Ogundero [17] reported that six thermophilic fungi produced amylases extracellularly in media with carbon sources other than starch but the highest amount of amylase was produced in media containing starch. The ability of some fungi to produce amylases in media containing other carbon sources apart from starch may be genetical or due to some cultural factors.

Various workers have reported on the ability of *B. theobromae* to breakdown the carbohydrate content, mainly starch, of various tubers and root crops during the pathogenic process [7, 11, 12]. The amylolytic nature of *B. theobromae* and *S. rolsii* as demonstrated in this study coupled with the ability to degrade starch in the host tissue shows the role of the two organisms in the degradation of cocoyam tissue.

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STAPHYLOCOCCUS AUREUS AND STAPHYLOCOCCUS EPIDERMIDIS STRAINS DIFFER IN INTERLEUKIN 2 INDUCING ACTIVITY

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Heat-killed preparations of different *Staphylococcus aureus* and *Staphylococcus epidermidis* strains were compared for interleukin 2 (IL-2) inducing activity in cultures of human mononuclear cells (MNC). *S. aureus* strains exhibited a strong individual variation, but they induced considerably more IL-2 than did *S. epidermidis* strains. Enterotoxin production, coagulase production, pigment formation and the phage type of the staphylococci showed no strict correlation of any of these properties with the IL-2 inducer activity. The results indicate that the IL-2 inducing activity may be a differential feature of *S. aureus* and *S. epidermidis*.

Staphylococci, their subcellular constituents and extracellular products can stimulate the various cellular elements of the immune system [1–6]. It has also been reported that staphylococcal enterotoxin A (SEA) induces IL-2 production in cultures of human mononuclear cells (MNC) [7]. On the basis of the IL-2 inducing activity of SEA, we have recently applied a heat-killed preparation of a *Staphylococcus aureus* strain for the stimulation of IL-2 production in cultures of human MNC [8]. We found that the IL-2 production of the *S. aureus*-stimulated MNC was comparable to those induced by either concanavalin A or SEA. With a possibly more efficient inducer in mind, it seemed us to be of interest to compare the IL-2 inducing activities of other strains of staphylococci.

Materials and methods

Bacteria and their characterization. The staphylococcal strains with known enterotoxin production were kindly provided by Dr. E. Marjai (Csongrád County Public Health Station, Szeged, Hungary). The other staphylococcal strains were isolated from clinical specimens and were provided by Dr. E. Nagy (Department of Clinical Microbiology, University Medical School, Szeged, Hungary). Phage typing and the determination of the haemolysis of bovine erythrocytes were carried out by Dr. J. Lantos (Csongrád County Public Health Station, Szeged, Hungary) [9]. The determination of free and bound coagulase was performed in our laboratory by the standard procedure [10]. Pigment production and haemolysis of rabbit erythrocytes were observed by visual inspection of colonies grown on nutrient agar and on blood agar plates containing 5% rabbit blood, respectively. The strains were denoted by serial numbers in combination with the first letters of their generic and specific names, i.e. SA-1, 2 etc. for *S. aureus*, and SE-1, 2, etc. for *S. epidermidis*.

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Preparation of heat-killed suspensions of bacteria. Staphylococci from single colonies were inoculated into brain heart infusion broth (BHIB), (Difco) and incubated for 10 h at 37 °C. At the end of incubation, the bacteria were washed twice and resuspended in PBS. They were killed by incubation at 90 °C for 10 min. The heat treatment was followed by another two washes with PBS, and the bacteria were resuspended at a concentration of 10^9 bacteria/ml. The effectiveness of heat treatment was tested by inoculation of 0.1 ml suspensions into BHIB and into thioglycolate medium.

IL-2 induction and assay. Heat-killed bacterial suspensions or their appropriate dilutions were added in 1/10 volume to cultures of human MNC isolated by centrifugation on Ficoll-Uromiro from individual healthy blood donors. Concerning the maximal amount of IL-2 activity produced, the optimal ratio of bacteria to MNC was determined by dilution of the bacterial suspension. MNC cultures were incubated for 14 h with the inducer, then centrifuged, and supernatants were assayed for their IL-2 activities on an IL-2 dependent murine cytotoxic T lymphocyte cell line (CTLL) and on mouse thymocytes determining their ^3H -thymidine (UVVR, Czechoslovakia) uptake as described previously [8].

Results

We determined the dose-response relationship of the inducer preparation of strain SA-1 for the optimization of induction. Different bacterium/MNC ratios were prepared by adding 1/10 volumes of various dilutions of the heat-killed SA-1 suspension to MNC cultures containing 5×10^6 cells/ml. The effectiveness of induction increased up to a ratio of 20 bacteria per MNC (Fig. 1). We therefore used this amount of inducer in further experiments.

In investigating the IL-2 inducing activities of different staphylococcus strains, we used MNC isolated from individual donors. Usually, 100 ml blood samples were obtained, and the MNC were sufficient for the testing of 10–15 bacterial strains in duplicate cultures. We observed a strong individual variation (up to a three-fold difference) in the IL-2 producing capacity of the MNC

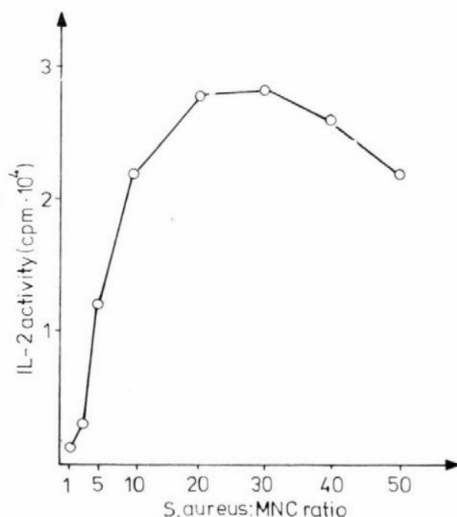


Fig. 1. IL-2 production of human MNC stimulated by different amounts of *S. aureus*

of the various blood donors. To overcome this disadvantage, we applied two kinds of measurement. Firstly, we included strains SA-1 and SE-1 in all tests. Secondly, we calculated the relative IL-2 inducing activity for the characterization of the different strains as follows. In each experiment we compared the amounts of IL-2 induced by different staphylococcal preparations with the spontaneous IL-2 production of MNC treated with a 1/10 volume of PBS only. The relative IL-2 inducing activity of a preparation therefore indicates how many times higher was the IL-2 activity detected in the MNC cultures induced by the particular inducer in comparison with that observed in the PBS-treated control. The quotients determined in the individual experiments were finally averaged for each staphylococcal culture and, depending upon the strain, the relative IL-2 inducing activity was given by the means of 8 to 20 determinations. The results, together with other characteristics of the investigated staphylococcus strains, are listed in Table I. The relative IL-2

Table I

Characteristics of S. aureus and S. epidermidis strains and their relative IL-2 inducing activities

Strain	Characteristic							Relative IL-2 activity inducing
	Enterotoxin	Free coagulase	Bound coagulase	Pigment	Haemolysis of rabbit RBC	Haemolysis of bovine RBC	Phage type	
SA-1	A+D	+	+	y	+	A	III	41.6
2	A	+	+	y	+	—	×	24.7
3	A+D	+	+	y	+	—	III	25.8
4	B	+	+	p	+	A	misc	23.2
5	—	+	+	y	+	A	misc	16.6
6	A	+	+	p	+	A	×	14.6
7	—	±	+	y	+	A	bovine	15.4
8	C	+	+	p	—	A	III	20.7
9	D	+	+	y	+	A	bovine	7.6
10	E	±	+	w	+	B	misc	21.7
11	F	+	+	p	—	—	misc	6.6
12	F	+	+	p	—	—	misc	6.7
SE-1	nt	—	—	w	—	—	nt	1.56
2	nt	—	—	w	—	—	nt	1.3
3	nt	—	—	w	—	—	nt	1.8
4	nt	—	—	w	—	—	nt	1.26
5	nt	—	—	w	—	—	nt	1.02
6	nt	—	—	w	—	—	nt	1.74
7	nt	—	—	w	—	—	nt	2.2
8	nt	—	—	w	—	—	nt	1.5
9	nt	—	—	w	—	—	nt	0.89
10	nt	—	—	w	—	—	nt	1.03

Enterotoxin: nt = not tested

Pigment: y = golden-yellow; p.=golden-yellow (pale); w = whites

Haemolysis of bovine RBC: A = complete β -type haemolysis; B = hot-cold lysis only

Phage type: misc = miscellaneous; nt = not tested; x = not typable

inducing activities ranged from 6.7 to 41.6 for *S. aureus*, and from 0.89 to 2.2 for *S. epidermidis*.

Discussion

The IL-2 inducing activity of a staphylococcal strain can be due either to its nonspecific stimulant activity for T cells, similar to that of concanavalin A, or phytohaemagglutinin or to a specific commitment of the immune system towards that antigen. Our strains failed to agglutinate in the sera of the blood donors, which seems to exclude a recent humoral immune response to these antigens.

We examined the correlations of the IL-2 inducing activities of the strains with the other characteristics listed in Table I. Most of the best inducers (relative IL-2 inducing activity >20) produced at least one enterotoxin, haemolysed rabbit erythrocytes and produced coagulases. Neither of these nor other characteristics, however, exhibited a strict correlation with the IL-2 inducing activity, since they could also be found among the properties of relatively weak inducers, such as strains SA-6 and SA-9.

Interestingly, all of the tested *S. epidermidis* strains had a relative IL-2 inducing activity less than 2.5, and in 5 out of 10 strains the values were below 1.5. This seems to indicate a substantial difference between the IL-2 inducing activities of the *S. aureus* and *S. epidermidis* species. The higher activity of *S. aureus* may be characteristic of this species. To clarify this possibility, we plan to compare the IL-2 inducing activities of further species of staphylococci.

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LONG-TERM PRESERVATION OF THE ENTEROINVASIVE *ESCHERICHIA COLI* FACTOR RESPONSIBLE FOR EXPERIMENTAL KERATOCONJUNCTIVITIS

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The factor responsible for experimental keratoconjunctivitis (EKC) can be successfully preserved in enteroinvasive *Escherichia coli* (EIEC) strains for decades. In this respect an adequate freeze-drying technique is essential, whereas the question of protective media appears to be of less importance. The preservation of EKC-producing factor may be associated with the maintenance of some other related properties.

In 1924, Zoeller and Maoussakis [1] were the first to use experimental keratoconjunctivitis (EKC) as a test to demonstrate the pathogenicity of *Shigella dysenteriae* serotype 1 by instilling its viable culture onto the scarified cornea of rabbit eye. Independently of them, in 1955 Serény [2] obtained the same phenomenon with live *Shigella* strains in guinea pigs and rabbits with an intact cornea surface by a mere administration of the culture onto the conjunctiva. Further studies [3, 4] described the pathohistological picture of the guinea pig eye being consistent with the human eye pathology and the resulting tissue immunity. The same pathogenicity factor was described by Bulgarian authors [5] in shigella-like organisms later classified as enteroinvasive *Escherichia coli* (EIEC) strains. Because of the virulence factor identical with that of shigellae, EIEC strains have been proposed by some authors to be classified in the genus *Shigella* [5, 6]. The EKC test is a suitable tool for the isolation of shigellae or EIEC from heavily contaminated clinical material, and for the classification of strains difficult to identify [7]. The test is specific for both shigellae and EIEC. The EKC phenomenon produced by *Listeria monocytogenes* in the Anton eye test [8] is immunologically different. Salmonellae were found to produce conjunctivitis only, though Istrati et al. [9] discovered 7 strains with EKC potential among the cultures tested. Recently, plasmids were found to be the basic principle of EKC test [10, 11].

The generally accepted assumption [3, 12, 13] that only freshly isolated strains are capable of causing EKC initiated us to perform long-term studies on EIEC strains subjected to different preservation procedures.

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Materials and methods

Bacterial strains. The EIEC strains used in this study are summarized in Table I.

Methods of preservation. The strains of *E. coli* No. 6551 S (O124 : K72) was kept freeze-dried, and on nutrient preservation media. Freeze-drying was performed by suspending 18–20 h nutrient agar culture in the following media: L-1, saline; L-2, equal volumes of 10% aqueous lactose solution and calf serum; L-3, equal volumes of 20% lactose+2% gelatin in distilled water and calf serum; L-4, 6.0 g peptone, 0.5 g starch, 1.0 g gelatin in 100 ml distilled water, pH 7.2–7.4; L-5, same as medium 4, but supplemented with 1.0 g of sodium glutamate; L-6, 0.5 g cysteine HCl, 0.1 g tryptophan, 0.5 g lactose in 100 ml saline, pH 7.2–7.4; L-7, 10% solution of Skim Milk (Difco) in distilled water, pH 7.3. The suspensions were frozen with dry ice and lyophilized by the use of a Secfroid Switzerland freeze-dryer [14]. Freeze-dried cultures were tested throughout without rehyophilization. Data of freeze-drying are given in Table III. The cultures were kept also on nutrient media: (i) as stab cultures in nutrient agar medium enriched with 10% calf serum in tubes with rubber stoppers and overlaid with paraffin oil after growth; (ii) on Dorset egg yolk medium as slant cultures. The other strains were freeze-dried in medium L-2 or L-5.

Experimental keratoconjunctivitis test. According to Serény [2] a 18–20 h agar culture from a non-selective medium was instilled into the eye of guinea pigs (200–400 g). Readings were done after 24–72 h or after a week. Conjunctivitis developed mostly after 24 h, keratitis within 48–72 h or later.

Results

The studies were begun in 1964 with strain *E. coli* O124 : K72 (No. 6551 S), which had been maintained both freeze-dried and on nutrient substrates. The results of two decades' investigation are summarized in Table II. It is evident that during this 20-year investigation period all freeze-dried strains gave positive EKC test within 48 h irrespective of the suspending medium used, including saline (medium L-1). The batches on nutrient substrates retained EKC positivity for 6 years, but at the next checking (after 10 years) they were not viable.

Results for other EIEC serovars are shown in Table III. Except No. 24133 (O136 : K78) kept on Dorset medium, all strains were freeze-dried (medium L-2 or L-5).

Seven out of 18 strains were EKC negative; all these negative strains were received from foreign collections giving no information on isolation time, technique of preservation, or EKC-producing capacity at the time of acceptance (e. g. strains of serovar O112 : K66). The remaining 11 strains were EKC positive (for as long as 21 years).

Discussion

In terms of EKC test and subsequent immunity in the guinea pig eye there is no difference between shigellae and EIEC. This is why Manolov [5, 6], Trifonova [16] and Stenzel [17] proposed to classify the EIEC strains as a special subgroup of the genus *Shigella*. As revealed by DNA reassociation studies, DNA of both genera exhibit a high degree of homology between

Table I
Approved EIEC serovars and their origin

No.	Serovar			Designation of strain	Year of isolation	Date of freeze-drying	Origin
	O	K	H				
1	28ac	73	NM	M 207	1964	22.10.65	A. Trifonova, Sofia, Bulgaria; original denomination: <i>Shigella lom</i> , Ca 792, lactose-positive
2	28ac	73	NM	M 210	1964	9.11.71	Stools, Central Bohemia region; Czechoslovakia; <i>Shigella lom</i> , Ca 792
3	28ac	73	NM	145-11372	1966	2.12.66	Stools, Kirkuk, Iraq
4	112	66	NM	25477		11.6.84	M. R. F. Toledo, Sao Paulo, Brazil; named <i>Shigella guanabara De Asis</i> (15)
5	112	66	NM	25481	1955	13.6.84	NCTC 9704 "Ewing 1686"
6	112	66	NM	25487		13.6.84	I. Ørskov, Statens Serum Institut, Copenhagen, Denmark
7	124	72	30 (32)	EcK 181/59		24.10.63	F. Kauffmann, Statens Serum Institut, Copenhagen, Denmark, "Ewing 227", raffinose-negative
8	124	72	NM	6551 S	1963	12.2.64	Stools, Czechoslovakia; raffinose-negative
9	136	78	NM	24133	1982		Stools, Czechoslovakia
10	143	x1	NM	EcK 248/65	1958	12.11.73	B. Lányi, HNCMB, Budapest, Hungary + F. Kauffmann "4608-58"
11	143	x1	NM	197-11787		1.11.67	Stools, Prague, Czechoslovakia
12	144	x2	NM	EcK 249/65	1956	30.1.74	B. Lányi, HNCMB, Budapest, Hungary + F. Kauffmann "1624-56"
13	144	x2	NM	125-9828	1965	31.1.66	Stools, Ostrava, Czechoslovakia
14	152	?	NM	M 232		8.2.68	L. A. Trabulsi, Faculdade de Medicina, Sao Paulo, Brazil
15	164	?	NM	M 097		5.2.74	A. Trifonova, Sofia, Bulgaria; original denomination: <i>Shigella sofia</i> , serotype 147
16	164	?	NM	M 225	1946	21.2.68	K. P. Carpenter, PHLS, London, England; "145-46" serotype 147
17	164	?	NM	23663	1982		Stools, Czechoslovakia
18	164	?	NM	22282	1979		Stools, Czechoslovakia

NM = non motile

Table II*Results of long-term follow-up of EKC in EIEC strain 6551 S (O124:K72:H-)*

Suspending medium	EKC, years			
	1	5	10	20
L-1 saline	+	+	+	+
L-2 lactose 10%	+	+	+	+
L-3 lactose 20%	+	+	+	+
L-4 peptone	+	+	+	+
L-5 peptone + Na glutamate	+	+	+	+
L-6 cysteine HCl	+	+	+	+
L-7 skim milk	+	+	+	+
Serum agar stab culture	+	+	×	×
Dorset slant culture	+	+	×	×

× = non viable

Table III*EKC of 17 EIEC strains*

O	Serovar K	H	Designation of strain	Date of freeze-drying	Results of EKC 14.1.87	Period of pre- servation in areas
28ac	73	NM	M 207	22.10.65	neg*	22
28ac	73	NM	M 210	9.11.71	+	16
28ac	73	NM	145-11372	2.12.66	+	21
112	66	NM	25477	11.6.84	neg*	3
112	66	NM	25481	13.6.84	neg*	3
112	66	NM	25487	13.6.84	neg*	3
124	72	30	EcK 181/59	24.10.63	neg*	24
136	78	NM	24133	1982 ^a	+	5
143	x1	NM	EcK 248/65	12.11.73	+*	14
143	x1	NM	197-11787	1.11.67	+	20
144	x2	NM	EcK 249/65	30.1.74	+	13
144	x2	NM	125-9828	31.1.66	+	21
152	?	NM	M 232	8.2.68	+*	19
164	?	NM	M 097	5.2.74	neg*	13
164	?	NM	M 225	21.2.68	neg*	19
164	?	NM	23663	3.6.86 ^a	+	1
164	?	NM	22282	6.6.86 ^a	+	1

NM = non motile

^a = Preservation on Dorset medium

* = Strains from foreign collections (see Table I)

shigellae and EIEC [18, 19]. Ørskov in Bergey's Manual [19] quotes on this suggestion: "... many strains are phenotypically intermediate between *Escherichia* and *Shigella* but for obvious reasons a special taxonomic status for such strains is not warranted". Whatever the EIEC classification, it is important that these strains are as pathogenic as shigellae. The EIEC strains had shigelloid morphology and only 3 serovars (O29, O128 and O143) produced lactose-positive colonies. By their biochemical activity EIEC strains are intermediate between the genera *Escherichia* and *Shigella*.

The original results of EKC test for old collection cultures (*E. coli* serovars O112, O124 and O164) are unknown and, therefore no conclusion can be drawn from the negative findings in our experiment. Results with other strains indicate that the EKC factor must be fairly stable in view of its persistence in freeze-dried saline medium. Moreover, adequate preservation of the virulence factor responsible for EKC may be associated with the maintenance of other related, hitherto unrecognized properties. This may be particularly true for the preservation of epidemiologically important strains.

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FEEDING BY MUCIN AND INTESTINAL GROWTH OF SOME ENTERIC BACTERIAL PATHOGENS*

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Enteroinvasive *Escherichia coli*, *Salmonella typhi-murium*, *Shigella sonnei*, *Shigella flexneri*, as well as *E. coli* K-12 show dose dependent growth in minimal medium completed with purified hog gastric "Granular Mucin". This ability is based on alpha-galactosidase production: defective, melibiose (and galactose) non-fermenting K-12 mutant were unable to utilize mucin. The viability of the parent K-12 strain in the cecal content of mice is significantly higher than that of its Mel⁻ mutant phenotype. In mixed infections of mice the parent strain was the only one to be able to establish a monoflora against its Mel⁻ or Gal⁻ mutants. Among other mechanisms, the growing ability in the intestinal mucous layer may be an additional virulence factor when the enteric pathogens are exposed to a competitive antagonism of the normal flora.

For exerting a pathogenic process, in case of some enteric bacterial pathogens, e. g. enterotoxigenic and enteropathogenic *Escherichia coli* (ETEC, EPEC), intestinal adhesion and colonization are the basic requirements. On the other hand, surprisingly, the bacterial colonization of the intestine by *Salmonella*, *Shigella*, enteroinvasive *E. coli* (EIEC) and even *Vibrio cholerae* has not yet been proved. Freter et al. [1] suggested that *V. cholerae*, in contrast to the ETEC strains, does not colonize, but actively penetrating the mucous layer of the small intestine, multiplies in this mucus, which serves as energy source.

In 1982 Prizon [2] showed the in vitro growing ability of a strain of *Shigella flexneri* in purified mouse mucin. This capacity was associated with alpha-galactosidase activity supplying energy source by cleaving the terminal galactose of mucin molecules.

Our experiments were carried out on the basis of the above observations, taking into account that the alpha-galactosidase gene is generally present in the enterobacterial chromosomes [3, 4].

Materials and methods

Strains used are listed in Table I. The K-12 strain of J53 was kindly provided by N. Datta (London), while the *S. typhi-murium* strain by R. Helmuth (Berlin). The derivative J53 (pSPI) carrying the virulence plasmid of EIEC strain No.34 of O124 was constructed in this

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institute [5]. Fermentation mutants were induced by Methansulfonic Acid Ethyl Ester (EMS) according to Lazdunski and Shapiro [6]. The *E. coli* strain designated as "normal murine" was isolated from the faeces of a healthy mouse.

Media and chemicals. Luria broth and LB-agar complete media were used. Minimal medium was prepared according to Rothman and Corwin [7]. Fermentative characters were tested in 1% peptone water and on plates containing Endo agar base.

For mutagenic induction EMS (Sigma, St. Louis, USA), for the testing of alpha-galactosidase activity D-melibiose monohydrate (Sigma) and for purified, standard mucin a hog gastric mucin preparation (Granular Mucin, Type 1701-W, Wilson Labs., Chicago, USA) were used. In the experiments a heat-sterilized (100 °C, 30 min) 5% suspension of granular mucin was applied.

Animal experiments. CFLP outbred mice (Gödöllő, Hungary) were used. Essentially our "Mouse Shigellosis Model" [8] was used in a form of the "Relative Colonizing Ability" test modified according to Cohen et al. [9]. Mice were pretreated orally with 50 mg of streptomycin on two consecutive days. Each of the animals were placed in a sterile jar, housed in a sterile box and fed on autoclaved food. After checking for the absence of the aerobic bowel flora they were infected orally with about 10^3 germs. This dose was applied mostly also in the mixed infections. Because only virulent *Shigella* or EIEC strains can establish a long lasting carrier-ship [10], in the K-12 experiments the derivative J53 (pSPI) and its mutants were used.

Experiments for checking the intestinal viability of strains in mouse intestine were performed basically on the "Mouse Model" taking cecal samples for plate counts.

Results

1. *In vitro*, "mucin feeding" experiments. As it was shown in Table I, all the investigated wild-type strains of *E. coli*, *Shigella*, and *Salmonella*, including K-12(J53) fermented melibiose between 1–4 days. In one case the melibiose-induced LB culture supernatant of strain J53 was tested for human B blood group antigenic degradation (washed 5% suspension of RBCs and the culture supernatant were mixed at 0 °C and maintained at 30 °C for 4 h). In contrast to the control cells (treated with uninoculated medium), that showed an anti-B titre of 1 : 48, the culture supernatant treated cells had only an anti-B titre of 1 : 3.

Table I
List of strains

Designation	Character
<i>E. coli</i> K-12 J53	<i>met</i> ⁻ <i>pro</i> ⁻ Mel ^{+1*}
J53 (pSPI)	140 Mdal plasmid carrier derivative (Mel ⁺¹)
J53 (pSPI)/EMS/10	Mel ⁻ mutant phenotype
J53 (pSPI)/EMS/5	Gal ⁻ mutant phenotype
Enteroinvasive <i>E. coli</i>	
0124 No. 34	virulent, Mel ⁺⁴
0143 No. 2	virulent, Mel ⁺¹
0164 87	freshly isolated, avirulent, Mel ⁺¹
"Normal" murine <i>E. coli</i>	Mel ⁺¹
<i>Salmonella typhi-murium</i>	
353/82	Mel ⁺¹
<i>Shigella</i> :	
<i>S. sonnei</i> 87	freshly isolated, Mel ⁺³
<i>S. flexneri</i> 3 20780	Mel ⁺¹

* Mel⁺¹⁻⁴ = melibiose fermentation after 1–4 days

Table II

 "Mucin feeding" of enteroinvasive *E. coli* O164, O124, and O143 strains

Minimal medium completed by	O164/87		O124/No. 34		O143/No. 2	
	germ count	(%)*	germ count	(%)	germ count	(%)
0.2% Glucose	7.7×10^9	(100)	9.9×10^7	(100)	1.0×10^7	(100)
Granular mucin						
0.031%	3.0×10^8	(2.1)	.	.	.	.
0.062%	3.4×10^8	(2.5)	.	.	.	.
0.125%	1.0×10^9	(8.1)	3.3×10^7	(33.3)	1.1×10^7	(100)
0.25%	1.1×10^9	(7.3)	8.4×10^7	(84.8)	2.0×10^7	(200)
0.5%	2.5×10^9	(32.4)	1.2×10^8	(121)	6.4×10^7	(640)

* In percentages of the glucose complementation

. Not investigated

Table III

 "Mucin feeding" of a virulent *S. typhi-murium* strain

Minimal medium completed by	<i>S. typhi-murium</i> germ count	V "353/82" (%)*
0.2% Glucose	2.0×10^7	(100)
Granular mucin		
0.125%	7.2×10^7	(360)
0.25%	7.7×10^7	(385)
0.5%	9.7×10^7	(485)

* In percentages of the glucose complementation

The EIEC strains of serogroups O164, O143 and O124 exerted very different dose dependent growing ability in case of mucin complementation as compared to the effect of glucose (Table II).

The virulent *S. typhi-murium* yielded a higher plate count with mucin than with 0.2% glucose (Table III).

For *Shigella* strains the feeding ratio of mucin : glucose was between 52 and 666%. It should be noted that in mucin utilization there was no difference between an avirulent, phase II population of *S. sonnei* and the corresponding phase I cells (Table IV).

E. coli strain J53 and its virulence plasmid carrying derivative (J53/pSP1) also showed a dose-dependent limited growth in mucin (25–31% compared to glucose). On the other hand, its melibiose or galactose non-fermenting mutants had lost this ability (Table V).

2. *Mouse experiments.* Under experimental conditions the monoflora of shigellae and EIEC bacteria are rapidly eliminated from the small bowel of

Table IV
 "Mucin feeding" of *S. sonnei* and *S. flexneri* strains

Minimal medium completed by	<i>S. sonnei</i> /87				<i>S. flexneri</i> 3 "20780"	
	phase I		phase II			
	germ count	(%) [*]	germ count	(%)	germ count	(%)
0.2% Glucose	1.4×10^8	(100)	1.1×10^8	(100)	3.0×10^6	(100)
Granular Mucin						
0.125%	1.8×10^7	(12.8)	1.1×10^7	(10.0)	7.6×10^6	(253)
0.25%	5.2×10^7	(37.1)	3.2×10^7	(29.0)	9.6×10^6	(320)
0.5%	7.4×10^7	(52.8)	5.0×10^7	(45.4)	2.0×10^7	(666)

* In percentages of glucose complementation

Table V
 "Mucin feeding" of *E. coli* K-12 strain J53 and its derivatives

Strains	Germ count (%) [*]			
	Glucose	Granular mucin complementation		
		0.125%	0.25%	0.5%
J53	3.5×10^8 (100)	3.4×10^7 (9.7)	6.8×10^7 (19.4)	1.1×10^8 (31.4)
J53 (pSP1)	3.9×10^8 (100)	4.0×10^7 (10.2)	7.1×10^7 (18.2)	1.0×10^6 (25.6)
J53 (pSP1)/EMS/10 Mel ⁻	2.1×10^8 (100)	1.8×10^6 (0.8)	1.0×10^6 (0.4)	1.2×10^6 (0.5)
J53 (pSP1)/EMS/5 Gal ⁻	3.2×10^8 (100)	2.1×10^6 (0.6)	2.5×10^6 (0.7)	2.3×10^6 (0.7)

* In percentages of 0.2% glucose complementation

NB. The minimal medium was also completed by 20 µg/ml of methionine and proline

mice, slowly from the coecum (unpublished data) while in the colon the epithelial penetration and multiplication leads to a permanent reappearance of these pathogens. On the basis of these findings we studied the surviving rate and period in cecal samples of mice infected orally by the parent Mel⁺ strain J53(pSP1) and its Mel⁻ mutant J53(pSP1)/(EMS)2 < 10. The data summarized in Table VI show a slow diminishing tendency in case of the Mel⁺ parent strain, whereas a rapid decrease of its isogenic Mel⁻ mutant (Table VI).

The higher viability of the strain utilizing mucin suggested an ability of overgrowing the mutant in mixed infection. Such experiments carried out in the "Mouse Model" are shown in Table VII. Accordingly, the Mel⁻ and Gal⁻ mutants could establish a monoflora if they had been administered separately.

Table VI

In vivo growth of E. coli K-12 strain J53 (pSPI) Mel⁺ wild-type and its Mel⁻ mutant phenotype in the cecal content of mice

Hours after oral inoculation*	J53 (pSPI) Mel ⁺	J53 (pSPI)/EMS/10 Mel ⁻
4	7.4×10^4	4.1×10^2
12	5.1×10^4	1.0×10^2
24	1.0×10^4	$< 10^2$
48	4.0×10^3	$< 10^2$

* Mice were orally infected by 2.1×10^3 germs of the parent strain and 4.1×10^3 germs of the Mel⁻ mutant derivative. Mice were pretreated by streptomycin for the eradication of the aerobic bowel flora. Samples of cecal content taken after the oral infection at the above intervals from 3 mice each were suspended, diluted and plated on LB-agar

Table VII

The relative colonizing ability of the E. coli K-12 strain J53 "wild-type" and its Mel⁻ and Gal⁻ mutants in orally infected mice

Infected orally by	Carrier/Infected		
	J53 (pSPI) Mel ⁺	J53 (pSPI)/EMS/10 Mel ⁻	J53 (pSPI)/EMS/5 Gal ⁻
<i>Single infections (control)</i>			
J53 (pSPI)	10/10	—	—
J53 (pSPI)/EMS/10	—	3/5	—
J53 (pSPI)/EMS/5	—	—	4/5
<i>Mixed infections</i>			
J53 (pSPI)+J53 (pSPI)/EMS/10	9/10	0/10	—
J53 (pSPI)+J53 (pSPI)/EMS/5	12/15	—	0/15

Streptomycin pretreated mice free of aerobic bowel flora were infected orally with about 10^3 germs of a single culture (control) and with wild-type Mel⁺ parent strain plus one of its Mel⁻ mutants. Mice were housed under sterile environmental conditions. Faecal samples were taken 13–15 days after infection and plated on Mel-Endo, or Gal-Endo plates

On the other hand, it is also clear that in mixed infections the parent, mucin utilizing strain became predominant in the artificial bowel monoflora.

In contrast to the above experimental conditions, in Nature certainly there is a competition between alpha-galactosidase producing bacteria. In a next series of experiment, mixed infections were studied using Mel⁺ strains. An example is shown in Table VIII where one of the competing strain was a "normal" murine *E. coli* and the other *S. flexneri* 3. The only modification of the method was that the infective dose of *Shigella* was about ten times higher than of its *E. coli* partner. Both the *Shigella* and the *E. coli* strain of murine origin had the ability to establish a monoflora, but in mixed infection, surprisingly, the *S. flexneri* strain predominated in the bowel flora.

Table VIII

Relative colonizing ability of strains *S. flexneri* 3 and a "normal" murine *E. coli*

Orally infected by	Carrier/Infected	
	<i>S. flexneri</i> 3 Mel ⁺	"normal" murine <i>E. coli</i> Mel ⁺
<i>Single infections (control)</i>		
<i>S. flexneri</i> 3 "20780"	5/5	—
"normal" murine <i>E. coli</i>	—	5/5
<i>Mixed infection</i>		
<i>S. flexneri</i> 3 + murine <i>E. coli</i>	12/15	3/15*

* Few colonies only

Streptomycin pretreated mice free of aerobic bowel flora were infected orally with about 10³ germs of the *E. coli* strain and with about 10⁴ germs of *Shigella* strain separately (control) and with their mixed culture. Mice were housed under sterile environmental conditions. Faecal samples were taken 15 days after infection and plated on Lac-Endo plates

Discussion

The ability to grow in the intestine is evidently an essential feature of bacteria forming the resident, or accidental bowel flora. According to Freter et al. [11] most intestinal bacteria inhabit the thick (about 400 µm) mucus itself and very few are actually attached to the tissue surface. The intestinal growth may also be an important feature of enteric pathogenic bacteria in the earliest step of the infectious process before invasion happens, partly because this multiplication amplifies the infective dose, partly because it is necessary to counteract against the moving mucosal layer.

One of the most important source of energy serving this growth may be the mucin itself. Prizont [2] showed not only the growth of his *S. flexneri* strain in purified mouse intestinal mucin, but also its mechanism through the enzyme alpha-galactosidase. Our experimental results are in agreement with of Prizont, proving the feeding capacity of a purified hog gastric mucin and also the significance of the alpha-galactosidase activity in vivo. Concerning shigella infection, the early prediction of the Formal team [12] should be mentioned, who regarded the intestinal multiplication ability as a requirement of infectivity.

On the other hand, the alpha-galactosidase activity seems to be a general feature of *Enterobacteriaceae*. On the chromosome map of both *E. coli* K-12 [3] and *S. typhi-murium* [4], the *mel* gene was localized at position 93 min. This operon carries the structural cistrons of alpha-galactosidase and the TMG-(thiomethyl-galactosidase)-permease II. An other permease, TMG-I is encoded by the "y" cistron of the *lac*-operon. It should also be mentioned that all of our

strains were melibiose-positive. Therefore, this enzymatic activity should be accepted as virulence factor only in a broader sense.

Under natural circumstances enteric pathogens are exposed to the antagonism of the normal bowel flora, including *E. coli*. Its significance and the favourable effect of its elimination by antibiotics to increase the infectivity of salmonellae was demonstrated by Miller and Bohnhoff [13] and Peter [14]. Similar observations were made by Freter et al. on experimental cholera [15] and shigella infections [16], using animals pretreated with streptomycin. Applying this method we demonstrated [17] an effective antagonism between the resident and other *E. coli* strains — independently of origin, pathogenicity or antigenic structure. This effect was reflected also in the LD₅₀ value of salmonellae [18]. The resident *E. coli* monoflora caused a 4–5 log₁₀ exponents reduction in infectivity (IC₅₀) of shigellae in a long lasting carriership [8].

The mechanism of the antagonistic effect of the bowel flora is a complex, multifactorial phenomenon, but includes also, according to Freter [19], a competitive antagonism concerning the carbon source in the reduced intestinal environment. One question remains to be answered: are or are not the strains involved in this competition equal partners. Our data show a very variable effect of mucin feeding as compared to the effect of glucose, and also an inequality in the mixed infections, e. g. those carried out with *S. flexneri* 3 and an *E. coli* strain. These data suggest further research work in this field.

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PROPERTIES OF INTERFERONS PRODUCED BY DIFFERENT CELL POPULATIONS*

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It is known that alpha-MuIFN is coded by more than 10 genes, but for beta-MuIFN and gamma-MuIFN only one gene has been found responsible for coding of each type [1]. The differences in the physico-chemical properties and the functions of the interferons expressed by different genes is not yet clear, though it was found, that alpha-IFNs coded by different genes were different in their amino acid sequences by 10% [2]. Not enough information is available on the properties of interferons produced by different cell populations of leukocytes *in vivo* after the induction by any IFN inducer.

In view of comparing the different properties, we pursued different inducers of interferons, belonging to polyribonucleic acid complex (complex polyguanylic-polycytidilic acid-polyguacil), dsRNA from phage f_2 (lafarin), dsRNA from yeasts *Saccharomyces cerevisiae* (ridostin) and one low molecular weight IFN inducer, tilorone.

Materials and methods

Mice. White males weighing 10–12 g.

Cell cultures. Mouse fibroblast cells, L-929.

Virus. Encephalomyocarditis (EMC).

IFN inducers. Polyribonucleotides complexes (polyguacil, ridostin and lafarin) were injected intraperitoneally (5 mg/kg), amixin (tilorone) was introduced orally (200 mg/kg).

Chemicals. Trypan blue, 0.5 mg/kg; theophylline, 1.5 mM/mouse; hydrocortisone, 1.25 mg/kg, were injected intraperitoneally 48 h before the inducers.

IFN assay. IFNs were assayed using an L-cell -EMC (100 TCID₅₀) system by CPE. An internal standard mouse IFN, which was calibrated against an international reference mouse IFN (G-002-904-511), was included in each titration. Titres were expressed as the reciprocal of the last dilution of test (log₂) which inhibited CPE 50% as compared with virus control wells.

In vivo studies. The mice were inoculated with the different inducers mentioned above. The organs and the sera were collected at different time intervals after the introduction of the inducers. For the preparation of cells from organs, — the mice were bled, the thymus, the femur

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bone and the spleens were collected by standard techniques in M 199 medium [3]. The spleen cells were treated with 2 M NH_4Cl . The final cell concentration was adjusted to 10×10^6 cells/ml.

In vitro treatment. Cells were treated with the inducers and other chemicals and DEAE dextran was added (500 $\mu\text{g}/\text{ml}$). After standing for an hour, the cells were washed and cultured in the RPMI-1640 medium with 20% calf sera and 100 mM glutamine and 20 mM Hepes buffer. Cells were incubated for 24 h at 37 °C in the CO_2 incubator and the culture media collected and titrated. The interferons were checked for their stability to pH 2.0 and 56 °C.

Results and discussion

Serum interferons produced in response to the induction were different in their stability to the effect of pH 2.0 and 56 °C. Difference existed between "early" interferon (taken 5 h after induction) and "late" interferon (taken after 24 h). "Early" interferon induced by polyguacil, for example was less sensitive to acidic and temperature effect than "late" interferon which is acid sensitive but temperature stable (Table I).

Table I

Sensitivity of the interferon produced by spleen cells to pH and temperature

Inducer	"Early" IFN, U/0.1 ml		"Late" IFN, U/0.1 ml	
	pH 2.0	56 °C	pH 2.0	56 °C
Poly(G).poly(C)	93.5%*	93.5%	75.0%	93.5%
Ridostin	0%	0%	75.0%	75.0%
Lafarin	75.0%	75.0%	50.0%	87.5%

* Sensitivity is expressed in percentage of the original activity of IFN

Comparison of "early" and "late" serum interferons, induced by the inducers under study in mice treated with trypan blue (affecting the macrophage system), theophylline (stimulating the T-suppressors) and hydrocortisone (affecting the T-helper cells) showed that "early" interferon in all cases differed from the "late" interferon, not only at the level of production, but also in reacting differently to the effects on the macrophage system, T-suppressors and T-helper cells. At the same time the spleen cells produced interferons which differed in their physical and chemical properties from the serum interferons.

Polyguacil induced "early" interferon (acid- and temperature labile); ridostin-induced "early" interferon (acid and temperature stable) and the "early" interferon stimulated by lafarin occupied an intermediate position (Table II).

Table II

Properties of the "early" and "late" serum interferon induced in mice

Inducer	"Early" IFN, U/0.1 ml			"Late" IFN, U/0.1 ml		
	Untreated	pH 2	56 °C	Untreated	pH 2	56 °C
Poly(G).Poly(C)	160	160	160	1280	160	640
Ridostin (dsRNA)	850	640	320	320	320	10
Lafarin (dsRNA)	720	160	640	320	10	<10
Tilorone	320	320	160	160	640	160

Table III

Properties of interferon produced by spleen cells from induced mice

Pretreatment of mice	Treatment of IFN	Interferon U/0.1 ml					
		Poly(G).	Poly(C)	Ridostin		Lafarin	
		E*	L*	E	L	E	L
None	*	160	160	160	40	80	320
	pH 2	<10	40	160	10	40	20
	56 °C	<10	10	160	<10	20	80
Trypan blue	—	<10	<10	<10	<10	<10	<10
Theophylline	—	320	80	640	40	80	320
	pH 2	<10	20	<10	<10	20	20
	56 °C	80	80	<10	<10	20	80
Hydro cortisone	—	80	320	160	160	320	640
	pH 2	<10	20	40	80	10	10
	56 °C	80	160	10	10	40	160

* E = "early" interferon, L = "late" interferon

Injection of trypan blue in mice 48 h before induction lead to a complete loss of ability of the spleen cells to produce interferon in response to all the three inducers.

Stimulation of T-suppressors by theophylline, which has been shown to strengthen the proliferation of T-cells of the spleen [1, 4], especially the immature cells of the spleen, somewhat raised the "early" interferon levels, and affected the "late" interferon levels. The interferons produced under its influence were acid labile (Table III).

The interferon induced in the spleen cells in vitro was similar to that produced by the fibroblasts and was comparatively stable to the effect of pH and temperature (Table IV).

The removal of the sticking fraction of monocytes does not affect the levels of interferons produced by T- and B-cells of spleen in the process of cultivation. The monocytes of the spleens are not the basic producers of inter-

Table IV

Comparitive properties of interferons produced by in vitro induced spleen cells and cells L-929

Inducer	Interferon, U/0.1 ml					
	Spleen cells			L-929 Cells		
	Original	pH 2	56 °C	Original	pH 2	56 °C
Polyguacil	640	320	160	320	320	320
Ridostin	640	160	160	640	640	320
Lafarin	640	160	160	640	640	320

feron, as shown by human leukocytes induced by Sendai virus [2]. At the same time destruction of the normal functions of the macrophage system by trypan blue leads to a drop in the production of interferon by the spleen that is not dependent on the application of the inducer, which means an important role in the IFN synthesis in the spleen cells. Thus one must not exclude the possibility that macrophage system is necessary for the production of interferon by spleenocytes ("Three cell cooperation system", [5]).

Hydrocortisone failed to show any effect on the induction of interferon by the non viral inducers. The fact that under its influence the physical and chemical properties of interferon changes, may indicate that the change is related to the different types of interferons in the produced pool.

At the same time it has been shown that steroid hormones possess the property of interferon by cells of the peripheral blood and the spleen cells.

In vitro studies showed that not only spleen cells, but also, in a lesser degree, bone marrow and thymus cells can produce interferon. In vivo, ridostin induced interferon in the bone marrow cells 48 h after induction, but in the thymus cell cultures interferon was not detected before 72 h after induction (Table V). It was observed at the same time that the spleen cells induced in

Table V

Interferons produced by

Source of IFN	Inducers, time*				
	Poly(G).Poly(C)			Ridostin	
	5	24	48	5	24
Sera	320	640	40	640	640
Thymus	<10	40	40	<10	<10
Bone marrow	80	<10	<10	<10	<10
Spleen	80	80	<10	160	40

* Animals were induced their organs collected at different time intervals and the cells were cultivated for 24 h and m titrated for IFN

vivo produced an interferon different from the serum interferons in its physical and chemical properties.

Accordingly, cell cultures, derived from thymus, bone marrow and spleen, reacted differently to interferon induction, depending on the inducer and its application (in vivo or in vitro). It seems from Fig. 1 that only spleen cells were able to produce interferon 5 h and 24 h after the in vivo induction (ridostin, 5 mg/kg). Thymus derived cells practically did not produce interferon, though cells taken from normal mice and induced in vitro synthesized relatively high levels of interferon.

On the other hand, after a single dose of interferon inducer injected into the mice, all tested interferon-competent cells became resistant to the repeated stimulation in vitro. The hyporeactivity was detected at 5 h and it lasted up to 72 h.

In order to check whether the inhibitory effect of interferon production by the spleen cells would be accompanied by changes in the resistance to the viral infection, mice were pretreated with trypan blue, as well as with theophylline or hydrocortisone and infected with EMC virus. Theophylline and hydrocortisone yielded a slight protection (10 and 18% respectively), but trypan blue was ineffective. In some experiments the mice treated with trypan blue became more sensitive to viral infection, than the nontreated mice. In general, the pretreatment with trypan blue, theophylline or hydrocortisone did not change much the antiviral activity of the inducers used (Table VI).

All experimental results showed that different types of interferon were produced by different cell populations of B and T lymphocytes and similarly by macrophages. It was also shown that different types of T and B cells were induced during the induction of interferon by the dsRNA's and tilorone.

Trypan blue dye is known to be actively endocytosed and stored in the lysosomes of the macrophages and to inhibit the lysosomal hydrolases of the macrophages thus destroying their functions at certain concentrations. The

different cell populations

Inducers, time*						
Lafarin				Tilorone		
48	5	24	48	5	24	48
<10	640	640	<10	640	640	80
40	40	<10	<10	160	640	320
160	80	40	<10	640	640	640
40	80	80	<10	640	640	640

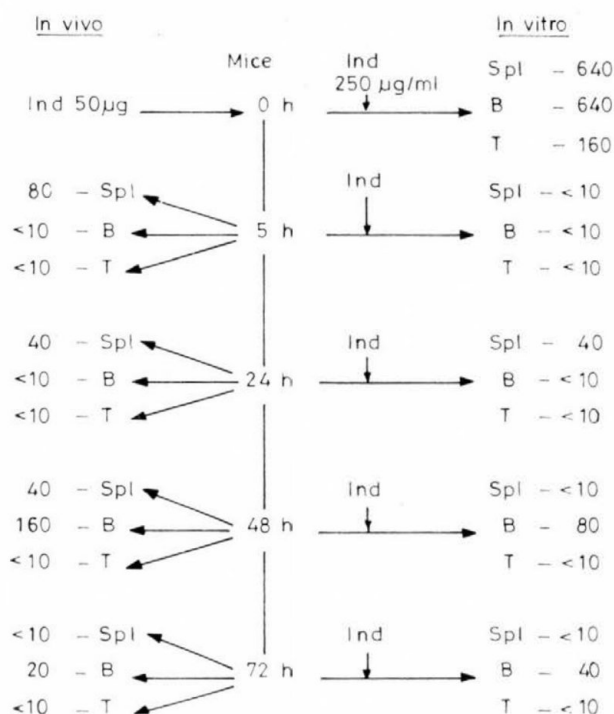


Fig. 1. Interferon production by different cell populations after induction in vivo and repeated induction in vitro with dsRNA. Interferon inducer (dsRNA), 50 µg/mouse, was given intraperitoneally. Spleen, bone marrow and thymus cell cultures were prepared 5, 24, 48 and 72 h after induction. Cell cultures were divided in two sets, one being treated with inducer (250 µg/ml) in vitro and cultivated for 24 h, the other being only cultivated for 24 h at 37 °C. Left side: IFN produced by cells taken from induced mice and cultivated for 24 h. Right side: IFN produced by cells having had additional contact with dsRNA in vitro. Spl = spleen cells; B = bone marrow cells; T = thymus cells; Ind = dsRNA

Table V I

Effect of trypan blue, theophylline or hydrocortisone on the antiviral activity of the interferon inducers in mice

Pretreatment of mice	Inducers*			
	Control	Ridostin	Lafarin	Poly(G).Poly(C)
No pretreatment	—	36.6%	52.8%	56.6%
Trypan blue	3.3%	42.4%	44.3%	63.3%
Theophylline	18.3%	35.5%	51.5%	63.3%
Hydrocortisone	10.0%	23.6%	48.6%	53.3%

* Mice were infected 24 h after administration of the inducer; the rate of protection is expressed in percentage

inhibitory action of trypan blue on the production of interferon by spleen cells, without affecting much the antiviral effect induced by interferon inducer, requires further explanation.

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EFFECTS OF INTERFERON AND TUMOUR NECROSIS FACTOR ON DEVELOPMENT OF INTRACELLULAR BACTERIAL INFECTIONS*

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Invasiveness of the epithelial cells in the gut by *Salmonella*, *Shigella*, enteroinvasive *Escherichia coli* and other facultatively intracellular enteropathogenic bacteria, is an important pathogenetic factor in the course of infection. This process is inhibited when cells are pretreated with different types of interferons as demonstrated in animal and in culture models. The tumour necrosis factor seems to have minor effect on this process. Mostly the internalization process is reduced, and, to a lesser degree, there is a decrease in adhesiveness to the cell surface. The internalization process is dependent on active cell metabolism. Interferon effect was not expressed when the cultures were treated with protein synthesis inhibitors. Shiga toxin inhibited the anti-invasive effect of interferons in toxin-susceptible cells.

This study was initiated on the basis of the following earlier observations. Interferon is often present in the organism during infection with various non-viral agents [1]. The presence of interferons can influence both specific and general functions of cultured cells. Some of these cellular functions may participate in the total host defense against infection with viral and non-viral agents. Several reports indicate that interferon treatment may alter the course of infection caused by such agents, first of all obligate or facultatively intracellular bacteria, protozoa and possibly some fungi [2–6]. The mechanisms of these effects are partially explained by alteration of host defense activities such as cellular and humoral immune response and phagocytic and killing activities of monocytes/macrophages and granulocytes [7–9]. In addition, interferons may influence the interaction between target cells and the invading microorganisms.

An essential process in the pathogenesis of infections produced by a major group of enteropathogenic Gram-negative rods is invasion of gut epithelial cells and establishment of an intracellular infection. This is followed by intracellular multiplication and destruction of the epithelial cells. *Salmonella*, *Shigella*, enteroinvasive *Escherichia coli* (EIEC) and possibly *Yersinia enterocolitica* belong to this group. The invasiveness of these bacteria can be examined in several in vivo models. More recently it has been shown that the ability to

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invade intestinal cells in the organism is well correlated with their ability to enter cultured cells in an *in vitro* system [10]. Such model systems are well suited for the investigation of factors that might influence bacterial invasiveness into cells. In a series of studies [2, 11–15] we have examined the effect of interferons (IFNs) and tumour necrosis factor (TNF) on this process. In this communication we present some of our recent findings.

Materials and methods

Bacteria. The following bacteria were included: *Salmonella typhi-murium*, *S. paratyphi-B*, *Shigella flexneri*, *S. sonnei*, *Yersinia enterocolitica*, *Escherichia coli* (EIEC). The origin and characterization of the strains used has been described in detail earlier [11, 13]. The bacteria were generally grown on chocolate agar and suspended in PBS to an optical density of ca 0.75 at 520 nm, corresponding to 3.5×10^8 bacteria per ml.

Cells. Monolayers of HEP-2 and A-549 human epithelial cell lines, or the L-929 mouse cell line were grown on glass coverslips in 24-well tissue culture plates. When the cultures formed an almost continuous monolayer, usually after 2–3 days of incubation, they were subjected to treatment with IFNs or other agents, usually overnight and then inoculated with bacteria. In some experiments treatment was applied at the same time as bacterial inoculation. Untreated control cultures were included in all experiments.

Invasiveness experiments. Monolayers of cells, inoculated with bacteria, were incubated for 3 h, and washed to remove bacteria not penetrated into the cells. In some cases the cultures were incubated for an additional 2 h to increase invasiveness. The cells were fixed overnight in glutaraldehyde, stained with acridine orange, and examined microscopically with a combination of Nomarski differential interference contrast microscopy and UV incident light microscopy, applied on the same microscope [16]. This system allows discrimination between extracellular and intracellular bacteria. The number of intracellular bacteria was counted in at least 200 cells.

Other cultures were not fixed, but exposed to treatment with gentamicin to inactivate extracellular bacteria. Thereafter the cells were lysed by addition of distilled water and the number of intracellular bacteria was estimated by counting colony forming units after plating appropriate dilutions on blood agar plates.

Interferons. Partially purified human leukocyte interferon was obtained from Dr. K. Cantell, Helsinki, recombinant human alpha-A interferon from Dr. S. Pestka, Nutley, N. J., partially purified mouse fibroblast interferon from the late Dr. K. Paucker, Philadelphia, P. A. and partially purified human interferon-gamma from Dr. J. Georgiades, Houston, T. X. Recombinant tumour necrosis factor was given to us by Dr. C. W. Czarniecki (Genentech), San Francisco, C. A.

Results

Our first observation was that if *S. typhi-murium* was added to HEP-2 cultures treated with homologous leukocyte interferons, its invasiveness was reduced compared to that in untreated control cultures (Table I). This effect was dose-dependent, with maximum at 100 units per ml with natural leukocyte IFN and 500 units per ml of recombinant alpha-A interferon. Both the proportion of cells containing bacteria and the number of bacteria per infected cell were reduced by up to 95%. This effect was neutralized by anti-interferon serum, proving its specificity. Mouse interferon did not influence the invasiveness in human cells.

In order to clarify whether the inhibitory effect was a general phenomenon we have tested other invasive *Enterobacteriaceae*. IFN treatment of

Table I*Effect of interferon treatment on invasiveness of S. typhi-murium in HEp-2 cells*

Interferon concentration (units/ml)	Per cent cells without bacteria	No. of bacteria per infected cell	No. of bacteria per 100 cells
0	68	5.0	160
50	66	4.3	110
100	97.5	2.3	6
500	93	2.9	22
1000	94	5.9	36
10000	81	3.5	68

HEp-2 cells did in fact inhibit invasiveness of *S. paratyphi-B*, but not that of *Y. enterocolitica*, *S. flexneri* or EIEC. Thus it seems that the mechanism(s) by which these bacteria enter the cells is different from that of *Salmonella* species. Alternatively, the inhibitory activities of interferons are neutralized or eliminated by products of *Shigella* or *Yersinia* cultures. On the other hand, human IFN inhibited invasiveness in A-549 cells, murine IFN did effectively inhibit establishment of intracellular infection in murine L cells by *S. typhi-murium*, indicating that the phenomenon is not limited to the HEp-2 cells. The activities of natural leukocyte IFN and the recombinant IFN- α -A were comparable. IFN- γ had a maximum effect at 10 units per ml, that is about ten times more effective than type 1 interferons related to their antiviral activities. The maximal effect of interferon- α was comparable to that of interferon- γ .

Two observations were of great value in order to investigate the apparent difference between effect on some but not on other Gram-negative rods. Firstly, Niesel et al. [17] reported that in cells inoculated with low number of bacteria and incubated for short time, IFN inhibited invasiveness also by *Shigella*. Secondly, we found that treatment blocking the cellular protein synthesis at the ribosomal level, e. g. by cycloheximide, eliminated interferon effect. Therefore we have tested whether Shiga toxin, an inactivator of the 60S ribosomal subunit, may influence interferon effect. In fact, treatment of HEp-2 cells with purified Shiga toxin, even in very low concentrations, completely eliminated the interferon effect on invasiveness of *S. typhi-murium* (Table II). Other inhibitors of protein synthesis like cycloheximide and abrin had similar effect. Finally, IFN effectively inhibited invasiveness of *S. flexneri* in A-549 cells, that lack receptors for Shiga toxin, to the same extent as of salmonellae.

Preliminary data indicate that if HEp-2 cells are pretreated with TNF, invasiveness of *S. typhi-murium* is reduced, but this reduction is generally less than that achieved by IFNs (Table III). This effect is dose dependent. In cells treated with IFN- α and TNF in combination invasiveness was inhibited to

Table II

Effect of protein synthesis inhibitors on invasiveness of S. typhi-murium in HEp-2 cells treated with interferon

Interferon (units/ml)	Protein synthesis inhibitor	Per cent cells without bacteria	No. of bacteria per infected cell	No. of bacteria per 100 cells
Experiment 1				
0	0	29	7.8	553
500	0	77	4.5	100
500	Shiga toxin (0.0001 µg/ml)	20	8.0	643
500	Abrin (10 µg/ml)	25	7.9	592
Experiment 2				
0	0	60	3.5	140
500	0	94	2.3	14
500	Cycloheximide (10 µg/ml)	62	3.4	129

Table III

Effect of treatment with interferon and tumor necrosis factor on invasiveness of S. typhi-murium in HEp-2 cells

Treatment	Intracellular bacteria (c. f. u. per well)
Interferon-alpha (500 µ/ml)	38*
Tumour necrosis factor (0.1 µg/ml)	28
IFN+TNF	35
Interferon-gamma (10 µg/ml)	56
Tumour necrosis factor (0.1 µg/ml)	25
IFN+TNF	61

* Per cent reduction compared to untreated control cells
(mean of 3 experiments)

the same extent as by IFN-alpha alone. On the other hand, if TNF treatment was combined with IFN-gamma an additive, in some experiments synergistic effect was observed.

Discussion

Our data indicate that interferons may participate in the pathogenesis of infection with facultatively intracellular bacteria at the level of direct interaction between the invading bacteria and the target cells. IFN alpha and gamma, in concentrations that can be encountered in the organism, reduce the

establishment of intracellular infection in an in vitro cell culture model. Comparable effect was seen in several cell lines and several bacterial species, indicating that the phenomenon is not restricted to one particular cell type of bacterial species.

The mechanism of this action is not quite clear. The process of intracellular infection includes several phases that may be influenced by IFN treatment, reversible and irreversible adhesion, endocytosis and intracellular multiplication. Some of our observations [15, 18] indicate that IFNs can reduce adhesion of several bacteria, as *S. typhi-murium* and *Pseudomonas aeruginosa* to cells in an in vitro system. The extent of this effect, however, seems to be much too small to explain the more marked effect on bacterial invasiveness. Indirect evidence indicates that the major effect is on the endocytosis phase. Like endocytosis, the effect on intracellular infection is dependent on active metabolism and protein synthesis [15]. Also earlier data indicate that IFNs alter the penetration of cell membrane by viral particles [19] and soluble substances [20]. At the present time we have no convincing data on IFN effect on intracellular multiplication of bacteria, although some observations [21, 22] indicate that such an activity might be present in some cases.

It also remains to be evaluated whether the in vitro phenomenon has any relevance in the infectious process in the organism. It seems to be clear that IFNs may be present both systematically and locally during bacterial infections, either induced by the bacteria or by a preceding virus infection [1]. It has been shown that IFN alpha or gamma administered before bacterial inoculation reduced mortality of mice inoculated with *S. typhi-murium* [2, 3]. The intestinal epithelium cells of the pretreated animals contained fewer bacteria than that of control animals. Similarly, we know that TNF is present during bacterial infections in the organism. Its role, and the possible modification of IFN effect(s) remains to be clarified.

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INHIBITED INTERFERON PRODUCTION AFTER SPACE FLIGHT*

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Several studies have been performed in our laboratories indicating that interferon production may be impaired in rodents after space flight. Using an antiorthostatic suspension model that simulates some of the effects of microgravity seen during space flight, we have shown that interferon-alpha/beta production was inhibited. The inhibition was not due solely to the stress of suspension. The inhibited interferon production was transient, as suspended animals returned to normal caging recovered the ability to produce interferon. Antiorthostatic suspension of mice also resulted in a loss of resistance to infection with the diabetogenic strain of encephalomyocarditis virus, which correlated with the drop in interferon production. In rats flown in US Space Shuttle mission SL-3, interferon-gamma production was inhibited severely when spleen cells were challenged with concanavalin-A upon return to earth. In contrast, interleukin-3 production by these cells was normal. These results suggest that immune responses may be altered after antiorthostatic modeling or space flight, and the resistance to viral infections may be especially affected.

Minor problems with respiratory tract infections and viral gastroenteritis have been reported in astronauts upon return from space flight [1]. As a result of that, interest has begun to be focused on the effects of space flight on immune response.

Over the past several years there have been reports of alterations in immunological parameters of humans and animals after space flight [2–7]. These have included alterations in size and functions of immunologically significant organs and suppression of cell-mediated immune responses. Very little significant effect of space flight on antibody responses has been reported [8].

One of the immune responses that has proven to be of interest is the production of interferons. Interferons are a group of cytokines produced by

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cells of the body [9]. They were originally described as antivirals, but now it appears that they play a major role in regulating immune responses [9]. Early studies were carried out by Tálas et al. [10–12]. They showed that leukocytes of two of four cosmonauts were inhibited in their production of interferon-alpha/beta when the leukocytes were obtained immediately after return to earth [10–12]. The inhibition was transient, and the other two cosmonauts showed no depression of interferon production [10–12]. On the other hand leukocytes placed in culture and challenged to produce interferon-alpha/beta in space showed enhanced interferon production [10–12]. These data suggested that there could be altered interferon production as a result of space flight, but the number of experimental subjects was very limited.

We began studies in animals to determine the effects of space flight on interferon production. We expanded the data base and the parameters tested. Our studies included both antiorthostatic modeling using mice and rats [13–18] of microgravity occurring during space flight, and actual flight experiments with rats [19]. These experiments and their results will be discussed in this review.

Materials and methods

Animals. For suspension experiments, male Simonsen (Gilroy, CA) albino rats 8–12 months of age weighing approximately 750 g or female Swiss mice 9 weeks of age weighing 25–30 g (Lab Supply, Indianapolis, IN) were used [15, 17, 18]. For space flight studies, specific pathogen free male Sprague-Dawley rats (Taconic Farms, Germantown, NY) were used [19]. Two weight groups of rats were used for flight studies, one 202–269 g, and one 367–421 g. No differences in results related to weight were observed [19]. All animals were maintained under the direct supervision of a veterinarian under US National Institute of Health guidelines.

Suspension procedures. Rats were lightly anaesthetized with ether, and a small ring of orthopaedic casting material was placed around the tail [15]. This was attached to a suspension apparatus as described previously [13] so the rats were at a 30° head-down tilt (antiorthostatic orientation) and could pull themselves around a grid with their front paws. For mice, a suspension system involving ultrasuede suits was used [16]. In this case, the mice were anaesthetized lightly with Rompin, placed in the ultrasuede suit, and attached to the apparatus. The mice were either oriented orthostatically (parallel to the horizon) or antiorthostatically (15–20° head-down tilt) were allowed to feed and drink at will.

Space flight procedures. The rats were flown in US Space Shuttle SL-3 for 7 days [19]. A 12 h delay after landing prior to obtaining samples was required to transfer the animals from the landing site to the laboratory [19]. The rats were sacrificed, their spleens removed, and teased into individual cells in RPMI-1640 medium with 10% fetal bovine serum (GIBCO Laboratories, Grand Island, NY). Matched control animals were housed in normal caging [19].

In vivo interferon induction. Interferon-alpha/beta was induced in rats by intravenous injection of 20 µg of annealed polyriboinosinic-polyribocytidylic acid (P-L Pharmacia, Milwaukee, WI) [15]. For mice, 10 µg was used [17]. Rats were bled 4 h after injection, mice 6 h after injection [15, 17]. Sera were prepared and stored at -70 °C for interferon assay.

In vitro cytokine induction. Rat spleen cells were adjusted to a concentration of 3×10^6 /ml and 1 µg of concanavalin-A was added. The cultures were allowed to incubate for 48 h [19], and culture supernatant fluids were then harvested, and stored at -70 °C for interferon-gamma and interleukin-3 assays.

Cytokine assays. Samples were assayed for interferon by plaque or microplaque reduction assay on mouse L-929 cells using the Indiana strain of vesicular stomatitis virus as the test virus [17]. Samples were assayed for interleukin-3 activity by determining the incorporation of (³H)-thymidine into DA-3 cells, an interleukin-3 requiring cell line [19].

Virus. The D variant of encephalomyocarditis virus (EMC-D virus) was a gift of Dr. D. Giron, Wright State University, Dayton, OH. Mice were infected intraperitoneally with 800 plaque forming units (0.2 ml) of EMC-D virus [18].

Glucose tolerance test. Mice were injected intraperitoneally with a glucose solution [18]. One hour later, the mice were bled, sera were prepared and analyzed on a Beckman glucose analyzer [18].

Results

Effects of suspension on interferon production. When rats were suspended in an antiorthostatic orientation for two weeks and then challenged with polyribonucleosinic-polyribocytidylic acid, interferon-alpha/-beta production was severely inhibited (Table I) [15]. Mice suspended in an antiorthostatic orientation for one or two weeks also had inhibited interferon-alpha/beta production after challenge with polyribonucleosinic-polyribocytidylic acid (Table I) [17].

Table I

Effects of suspension on interferon-alpha/beta production

Species	Treatment	Effect on interferon production
Rat	2-Week antiorthostatic suspension	Inhibited compared to controls
Mouse	1-Week antiorthostatic suspension	Inhibited compared to controls
	2-Week antiorthostatic suspension	Inhibited compared to controls
	1-Week antiorthostatic suspension followed by 1 week normal caging	Recovery of production
	2-Week antiorthostatic suspension followed by 1 week normal caging	Recovery of production
	1-Week orthostatic suspension	No effect on production

The inhibited interferon production was transient as mice suspended antiorthostatically and then returned to normal caging for one week regained their interferon production capacity (Table I) [17]. Mice suspended in an orthostatic orientation had no significant changes in their ability to produce interferon-alpha/beta (Table I) [17].

Effects of suspension on susceptibility to EMC-D virus infection. Normal control female Swiss mice were resistant totally to infection with EMC-D virus (Table II) [18]. Mice suspended in an antiorthostatic orientation for four days and injected with EMC-D virus at the initiation of suspension showed a marked susceptibility to virus infection as indicated by abnormal glucose tolerance test levels in a majority of mice (Table II) [18]. Mice suspended in an orthostatic orientation and injected with EMC-D virus at the initiation of suspension showed complete resistance to EMC-D virus infection (Table II) [18].

Table II*Effects of suspension on susceptibility of mice to EMC-D virus infection*

Treatment	Effects on GTT levels (indicating infection)
Normal injected with EMC-D virus	Normal — not infected
1-Week antiorthostatic suspension injected with EMC-D virus	Abnormal — infected
1-Week orthostatic suspension injected with EMC-D virus	Normal — not infected

Table III*Effect of space flight on cytokine production*

Cytokine	Production compared to controls
Interferon-gamma	Inhibited
Interleukin-3	Normal

Effect of space flight on cytokine production. Spleen cells from rats flown in Space Shuttle SL-3 showed inhibited production of interferon-gamma compared to matched controls when challenged with concanavalin-A upon return to earth (Table III) [19]. On the other hand, interleukin-3 production by spleen cells from rats flown in Space Shuttle SL-3 was equivalent to that of spleen cells from matched controls (Table III) [19].

Discussion

Over the years, it has become apparent that there can be several changes in various physiological functions after space flight [2-7]. One of the functions that appear to be affected by space flight is the immune response [2-7].

A particular area of interest to our laboratory has been the production and action of cytokines. Since cytokines can play such a major immunoregulatory role [9], it is possible that changes in cytokine production or action induced during space flight could result in widespread immunological alterations.

One of the most interesting group of cytokines are the interferons. Originally described as antivirals, it is now clear that the interferons have wide bioregulatory and immunoregulatory functions [9]. Early studies that were very limited in number of samples and were performed by Tálas et al. [10-12] raised the intruding possibility that the production of interferon-alpha/beta could be inhibited in cosmonauts when their cells were challenged

after return to earth, but that individual cells challenged during flight showed enhanced production. This suggested that effects of space flight could be very pronounced when the entire human or animal, with dynamic interactions among different biological systems, was exposed to flight.

In our laboratory, we have been using animal models for the past several years to determine how the interferon system is affected by space flight. These studies have been reviewed in this monograph. One model we have been using involves antiorthostatic, hypodynamic, hypokinetic suspension [13, 14, 16]. This model has its limitations, but does mimic some of the effects of microgravity seen during space flight [13, 14, 16]. In addition, controls can be run utilizing orthostatic suspension to test for the effects of the stress of suspension on a biological system [13, 14, 16].

In our hands, both rats and mice that were suspended antiorthostatically showed inhibited interferon-alpha/beta production [15, 17]. The inhibition of interferon production appeared to be transient, as suspended animals regained the ability to produce interferon after return to normal caging [17]. In addition, female mice normally resistant to EMC-D virus (an interferon-sensitive virus), became susceptible to infection after antiorthostatic suspension [18]. Orthostatically suspended, control animals produced normal levels of interferon and remained resistant to EMC-D virus [17, 18], suggesting that factors other than the stress of suspension were involved in the inhibition of interferon production.

Rats actually flown in US Space Shuttle mission SL-3 showed inhibited interferon-gamma production when their spleen cells were challenged with mitogen after return to earth [19]. However, production of interleukin-3, another immunoregulatory cytokine, was unaffected by space flight [19]. These data suggest that the antiorthostatic suspension model may be useful to predict the effects of space flight on immune responses, and that any effects of space flight may be specific to particular types of immune responses. The interferon system may be extremely sensitive to space flight exposures.

The results of the studies suggest that the interferon system is affected by space flight. The full nature, the mechanism, and possible clinical significance of the effects of space flight on the interferon system will require extensive future studies.

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ANTIGENIC STRUCTURE OF MAMMALIAN ADENOVIRUSES STUDIED BY MONOCLONAL ANTIBODIES AGAINST BOVINE ADENOVIRUS TYPE 2

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The cross-reactivity pattern of eight monoclonal antibodies (MAbs) raised against bovine adenovirus type 2 (BAV2) hexon was studied by indirect ELISA using seven different adenoviruses. All of the eight MAbs reacted with the two BAV2 subtypes (A and B), while the other adenoviruses, human adenovirus type 1 (HAV1), ovine adenovirus type 3 (OAV3), BAV1, BAV4 and BAV8 gave different combinations of positive reactions. Based on the results of cross-reactivity there is a close antigenic relationship between the two different BAV2 subtypes. There is an antigenic relationship among the adenovirus types studied, including viruses of human and animal origin. One of the MAbs (IV. F5) seems to be type specific. Another MAb (BH5) detects a common epitope among adenoviruses of animal origin, and reacts even with BAV4 and BAV8.

Bovine adenovirus type 2 (BAV2) is a virus interesting for its ability of causing pneumoenteritis both in cattle and sheep though most adenoviruses are species specific. Its economical and epizootiological importance is also significant, since BAV2 is widespread in sheep and cattle stocks of large scale industrialized farms as well as in sheep under extensive management conditions [1, 2] and causes sometimes heavy losses among young lambs and calves. In a previous study BAV2 strains from cattle and sheep have been analysed and differences were detected in haemagglutinating properties and restriction enzyme cleavage maps of serologically indistinguishable strains [3]. These differences seemed to be sufficient to group BAV2 isolates into two subtypes with representative strains Nos. 19 (subtype A) and ORT/111 (subtype B). Subtype A viruses were detected exclusively in cattle, whereas subtype B infection was demonstrated both in cattle and sheep.

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Though the protein structure and antigenic properties of human adenoviruses have been rather well studied [4, 5], we know much less about other mammalian adenoviruses apart from results obtained with conventional sera. However, a variety of antigenic determinants have been found on the hexon molecules, of which the determinant common to mastadenoviruses is called genus-specific antigen. The non-human adenoviruses are classified by their host animal. For the nine serotypes of bovine adenoviruses a subdivision into two subgroups was suggested [6]. Bovine adenoviruses belonging to subgroup II (BAV4, BAV5, BAV6, BAV7 and BAV8) exhibit only trace amounts of the genus-specific antigen, i. e. there is no cross-reaction between them and the other mammalian adenoviruses. The two groups can be differentiated by agar gel precipitation, complement fixation and immunofluorescent tests. Previous studies have shown that the hexon is one of the viral proteins reacting in these serological tests. On the other hand, conventional sera raised against the intact adenovirus or the hexon molecule could not distinguish hexon carrying the common genus-specific determinant.

The purpose of the work reported here was to develop MAbs against a BAV2 strain (ORT/111) and apply them to gain further informations about the antigenically important protein structure of the adenoviruses.

Materials and methods

Viruses. No. 19 (BAV2 subtype A prototype strain), BAV 1 prototype strain, as well as BAV4 and BAV8 strains were provided by Dr. A. Bartha (Veterinary Research Institute of the Hungarian Academy of Sciences, Budapest). ORT/111 (BAV2 subtype B prototype strain) was isolated in 1973 during an outbreak of pneumoenteritis of lambs on a Hungarian farm [8]. Ovine adenovirus type 3 (OAV3) prototype strain was obtained from Dr. J. B. McFerran (Veterinary Research Institute, Stormont, Belfast, Northern Ireland). The virus strains were propagated in secondary lamb testicle cell cultures. Virus particles were purified by equilibrium density gradient centrifugation using preformed CsCl gradients (1.1 to 1.4 g/ml) in 0.01 M Tris-HCl pH 7.5. CsCl was removed from the virus containing fractions by dialysis against PBS using Medicell dialysis membranes.

HAV1 virion suspension purified by CsCl equilibrium centrifugation was provided by Dr. Gy. Berencsi (Institute of Microbiology, Semmelweis University Medical School, Budapest).

Preparation of monoclonal antibodies. Spleen cells harvested from Balb/c mice immunized with ORT/111 virus were fused with Sp2/0 myeloma cells [9] using PEG 4000. Cells were treated and harvested as described by Van Deusen and Whetstone [10]. Ascitic fluid was produced by injecting approximately 5×10^6 hybridoma cells in 0.5 ml of serum-free medium into the peritoneal cavity of Balb/c mice. Mice were killed by cervical dislocation and ascitic fluids were harvested 10–14 days after inoculation.

Indirect ELISA. Assay of ORT/111 specific antibodies in the sera of immunized mice, in the hybridoma supernates and in the ascitic fluids were tested by indirect ELISA in Cooke microtiter plates coated with 10 µg/100 µl ORT/111 antigen. Cross-reactivity of the MAbs was studied with seven other adenoviruses in a similar system. The bound MAbs were detected with peroxidase-labelled rabbit anti-mouse immune sera (Human Institute for Serobacterial Production and Research, Budapest) using o-phenylene-diamine (OPD) as enzyme substrate. The reaction was stopped by adding 4 N H₂SO₄. The absorption was recorded at 492 nm using a Multiskan Titertek automatic ELISA plate analyser (Flow Laboratories, Paris, France).

Haemagglutination inhibition (HAI), virus neutralization (VN) and agar gel precipitation (AGP) tests were carried out as described by Belák et al. [3].

Polyacrylamide gel electrophoresis (PAGE) methods described by Laemmli [11] were used for separation of ORT/111 virion polypeptides. The electrophoresis was carried out with 10 V/gel-cm power in 10% slab gels. Gels were stained with Coomassie Brilliant Blue (CBB).

Electroblotting was performed on Biometra-Fast Blot apparatus (Biometra biomedizinische Analytik GmbH, Göttingen, GFR) at 5 mA/cm power. Proteins blotted onto nitrocellulose filter (Schleicher-Schüll, GFR) were renatured by soaking the filter in renaturing buffer (50 mM NaCl, 10 mM Tris-HCl pH 7.0, 2 mM EDTA-Na, 4 M urea and 0.1 mM dithiothreitol) for 45 min. The nitrocellulose stripes were soaked and saturated for 2 h at room temperature in ascitic fluids diluted in 1 : 100 ratio with PBS-Tween. After three washes with PBS-Tween the stripes were transferred into 1 : 100 diluted goat anti-mouse IgG and incubated for 1 h. The enzyme substrate contained 0.018% 4-chloro-1-naphthol (Serva, GFR).

Results

Monoclonal antibodies. Ten days after fusion the supernates of the clones were tested by ELISA and 21 clones out of 418 proved to be positive for specific antibodies. The test was repeated on the fourteenth day and 18 from the 21 previously positive supernates were positive again. The cells of these clones were transferred into the larger wells of T24 plates to allow expansion on the cultures. Eight of these clones showing highest absorption at 492 nm were recloned by limiting dilution and used for producing ascitic fluids. The clones were designated as follows: A12, BB7, BH5, CA12, ILA9, IILB11, IV. F3 and IV.F5.

None of the MAbs inhibited haemagglutination or neutralized the ORT/111 virus. In AGP tests MAbs BB7, IILB11 and IV.F3 precipitated different adenovirus types.

Specificity of the MAbs. Since the whole virus particle of ORT/111 strain was used for immunization and to develop MAbs, SDS-polyacrylamide gel electrophoresis and electroblotting were applied to determine the specificity of the MAbs. In PAGE the polypeptides of ORT/111 virus showed a pattern set out in Fig. 1. The molecular weights of the six major polypeptides visible in CBB stained gels were as follows: p1 = 101 000, p2 = 66 000, p3 = 55 000, p4 = 51 700, p5 = 34 500, p6 = 25 000. Polypeptides blotted onto the nitrocellulose filter were stained with aniline blue. All six bands were clearly visible. The greater part of the filter was cut in eight pieces and used for immunostaining. As seen in Fig. 2 all the eight MAbs proved to be bound to the polypeptide designated p1. Based on these results all the MAbs studied are specific to hexon molecule of ORT/111 virus.

Reactivity patterns of the MAbs. The cross reactivity pattern of the MAbs was determined by using seven different adenovirus strains. Two of them were the two subtypes of BAV2 (subtypes A and B). The remaining five virus types were BAV1, BAV4, BAV7, OAV3 and HAV1. The results are shown in Table I.

Two of the MAbs exhibited broad reactivity patterns. MAb IV. F3 reacted with the adenoviruses probably by their common genus specific epitope. MAb BB5 reacted with adenoviruses of animal origin, including

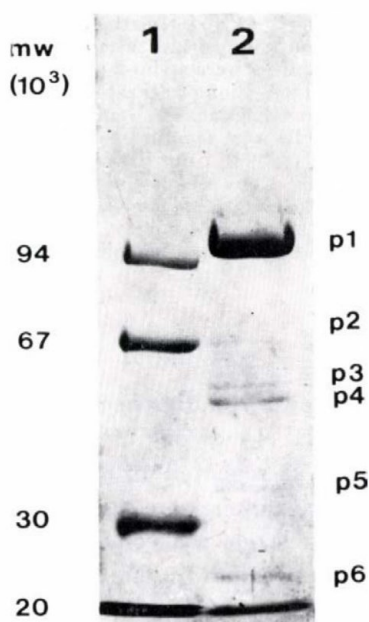


Fig. 1. The polypeptide pattern of ORT/111 (BAV2 subtype B) strain. (1) Molecular weight markers, (2) ORT/111 polypeptides

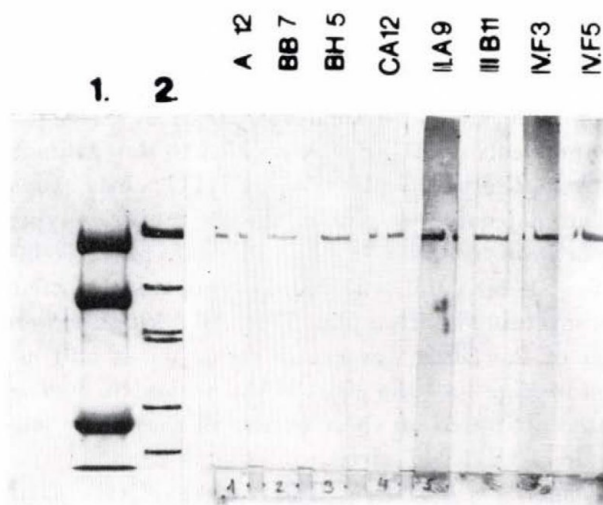


Fig. 2. Polypeptides of ORT/111 virus on nitrocellulose filter after electroblotting. The left part shows the strip stained with aniline blue, the right part the strips stained by immunostaining

BAV4 and BAV8, probably due to a common epitope on the hexon molecules of bovine and ovine adenoviruses. The other MAbs showed a variety of cross-reactivity patterns.

Table I

The ELISA cross-reactivity of different mammalian adenoviruses and monoclonal antibodies (MAbs) raised against BAV2 subtype B

Virus	MAbs							
	A12	BB7	BH5	CA12	IIA9	III.B11	IV.F3	IV.F5
BAV2/B	+	+	+	+	+	+	+	+
BAV2/A	+	+	+	+	+	+	+	+
HAV1	+	—	—	+	—	+	+	—
BAV1	—	—	+	—	—	—	+	—
OAV3	—	+	+	—	—	+	+	—
BAV4	—	—	+	—	—	—	—	—
BAV8	—	—	+	—	—	—	—	—

Discussion

Our original intention by using whole virus particles for immunization was to generate MAbs specific to BAV2 subtype B (ORT/111), and so to differentiate between the two subtypes of BAV2. Among the eight MAb-containing ascitic fluids, however, we have not found any to react exclusively with BAV subtype B. This is not astonishing, since the differences in the haemagglutination activity of the two subtypes suggest differences in the fiber structure, and immunostaining of the viral proteins proved that all our MAbs were generated against the hexon of ORT/111 virus. Our further studies were aimed to investigate whether the inability of the MAbs to differentiate the hexons of two viruses related to each other is regular or not.

The results of indirect ELISA experiments in which HAV1, BAV1 and OAV3 viruses, with detectable common antigenic determinant, as well as BAV4 and BAV8 viruses were used as antigens, suggest that epitope differences exist not only in the fiber but also in the hexon structure. According to the cross-reactivity pattern of the MAbs at least six different epitopes exist on the hexon molecules studied in these experiments. The range of the specificity of the MAbs extended from the probably genus specific recognition (MAb IV. F3) to type specificity (MAbs IIA9 and IV. F5). The other MAbs showed intertype specificities reacting with HAV1 (MAbs A12 and CA12), with OAV3 (MAb BB7) and with both BAV1 and OAV3 (MAb III.B11) beside BAV2 viruses. One of the MAbs (BH5) reacted with all adenovirus types of animal origin studied in these experiments. Since one of the MAbs could react with not only the bovine adenoviruses belonging to BAV subgroup I, but also with BAV4 and BAV8 belonging to BAV subgroup II, our result confirms the findings of Adair et al. [12] who demonstrated the presence of immunologically cross-reacting epitope on BAV subgroup I and II hexons.

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CHARACTERIZATION OF MULTIPLY RESISTANT *PROTEUS MIRABILIS* ISOLATES IN HUNGARY

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According to a survey on tendencies of antibiotic resistance in Hungary (1974–1983), the emergence of multiresistant (MR) *Proteus mirabilis* isolates is a new phenomenon. A total of 60 strains resistant to 11–16 antibiotics were collected from various specimens and geographical locations. Except for cefoperazone, third generation cephalosporins, amikacin, netilmicin and ofloxacin were effective in vitro against multiply resistant *P. mirabilis* isolates. Out of them 62% belonged to O18, a serogroup not shown in early studies in Hungary; moreover, in serogroup-distribution the present collection differed sharply from isolates examined in 1956. All but two strains produced the same aminoglycoside-modifying enzyme ANT (2''), and all of them proved beta-lactamase positive in nitrocephin test. All multiresistant strains harboured 2–4 plasmids, whereas the sensitive isolates had no plasmids. The presence of two plasmids (62 and 22 Mdal) occurring in 85% of the isolates was a characteristic feature of the multi-resistant *P. mirabilis* isolates. In contrast to these plasmids some other ones (17.5, 103 and 113 Mdal) could be transferred to *Escherichia coli* recipient. There was no relationship between plasmid profiles, serogroups and resistance patterns.

Infections caused by multiple-drug resistant bacteria have become increasingly common mainly in hospitalized patients. The organisms most often involved have been *Klebsiella* spp., *Enterobacter* spp., *Serratia* spp., indole-positive *Proteus* spp., and *Pseudomonas aeruginosa* [1, 2]. According to a survey on antibiotic resistance of most frequently occurring species in Hungary (1974–1983), *P. mirabilis* was the only one which showed an increasing resistance rate to all antimicrobial agents widely used in this country [3]. Emergence of *P. mirabilis* strains among the multiresistant Gram-negatives seemed to be a new phenomenon. Therefore the phenotypic properties and plasmids of these isolates were studied.

Materials and methods

Study strains. A total of 60 multiply resistant *P. mirabilis* strains isolated from different body sites in six clinical bacteriology laboratories in various geographical areas were collected in 1987. The term "multiply resistant" (MR) is used hereby to refer to strains which are resistant at least to the following antimicrobial agents usually employed for treatment of infections

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caused by *Enterobacteriaceae* in Hungary: ampicillin, carbenicillin, cephalexin, cefamandole, gentamicin, tobramycin, co-trimoxazole and tetracycline. Fifteen *P. mirabilis* strains showing sensitivity to many drugs originated from the Hungarian National Collection of Medical Bacteria.

Antibiotic sensitivity testing. Standardized disc diffusion and agar dilution methods were used [4]. Sensitivity of strains were tested to antibiotics listed in Tables I and II.

Serotyping. O serogroup of *P. mirabilis* strains was determined according to Kauffmann and Perch [5].

Beta-lactamase production. Growth from a nutrient agar plate was collected with wooden sticks. The sticks were dipped in 20 μ l nitrocephin (Glaxo) solution (500 μ g/ml) distributed in the wells of a microtiter plate. After thorough mixing, the colour change was recorded.

Identification of aminoglycoside-inactivating enzymes. A microbiological method described by van de Klundert et al. [6] was used.

Plasmid screening. Plasmid DNA was isolated essentially by the method of Portnoy et al. [7]. Agarose gel electrophoresis and visualization of DNA bands followed standard procedures [8]. Molecular weights of plasmid DNA were determined using standard plasmids of known molecular weights: V₅₁₇ [9], R1 and R27 [10].

Plasmid transfer. Plate mating was carried out according to Bradley et al. [11] using *E. coli* J53-2 as recipient [12]. After conjugation at 37 °C for minimum of 1 h or 24 h, suitable dilutions of conjugation mixtures were plated on agar containing the following antibiotics at the concentration indicated: ampicillin (50 μ g/ml), chloramphenicol (30 μ g/ml), gentamicin (25 μ g/ml), kanamycin (50 μ g/ml), tetracycline (25 μ g/ml) [8]. Repeated conjugation experiments were carried out with one or two representatives of strains belonging to different plasmid profiles.

Results

Isolation sites of the strains. The great majority (80%) of the isolates originated from urine samples. Sources of the remaining strains were as follows: trachea (6), throat (1), wound (2), blood (3).

Antibiotic resistance patterns. Resistance markers of the strains examined are shown in Table I. Sensitivity of the isolates was also tested to other cephalosporins, newer aminoglycosides and one quinolone derivative, ofloxacin. These drugs are available only in a limited amount in Hungary. Results are summarized in Table II. The MIC values for ofloxacin were elevated (~ 2 μ g/ml) comparing strains not being multiresistant. However, these values are lower than the sensitivity breakpoint stated for urinary isolates (16 μ g/ml).

Table I

Resistance markers of multiresistant *P. mirabilis* strains

Broad-spectrum beta-lactams	Aminoglycosides	Others
Ampicillin (Am)	Gentamicin (Gm)	Tetracycline (Tc)
Carbenicillin (cb)	Tobramycin (Tm)	Co-trimoxazole (Sxt)
Azlocillin (Azl)	Kanamycin (Km)	Chloramphenicol** (Cm)
Mezlocillin (Mz)	Neomycin (Nm)	Nalidixic-acid** (Na)
Cephalexin (Cl)	Streptomycin (Sm)	Oxolinic-acid** (Oa)
Cefamandole (Ma)		Nitrofurantoin* (Ni)
Cefoperazone (Cfp)		

* Occasionally moderately sensitive

** Occasionally moderately sensitive or sensitive

Table II*Drugs showing activity against multiresistant P. mirabilis isolates**

Second and third generation cephalosporins	Aminoglycosides	Quinolones
Cefuroxime** Cefoxitin** Ceftriaxone Cefotaxime Ceftazidime	Amikacin Netilmicin***	Ofloxacin

* Only the drugs listed in Tables I and II were examined

** Occasionally moderately sensitive

*** Only one resistant strain

Table III*Serogroup-distribution of multiresistant P. mirabilis isolates*

Serogroup	No. of strains	
	1987	1956*
O3	1	67 (23%)
O6	—	12
O10	—	22
O11	2	—
O13	—	27
O18	37 (62%)	—
O23	2	—
O26	5	22
O27	1	—
O28	—	24
O30	2	—
O42	1	—
NT	9	—
Other	—	122
Total	60	296

NT = nontypable

* Lányi [13]

Serogroup-distribution of strains is shown in Table III. The majority of isolates (62%) belonged to serogroup O18. The data were compared to serogroup-distribution of randomly selected *P. mirabilis* strains isolated in 1956 [13].

Antibiotic-inactivating enzymes. All strains proved beta-lactamase positive in nitrocephin test. All but two strains produced the same aminoglycoside-modifying enzyme ANT (2''). AAC(6,) II + APH (3,) and AAC(3)II + APH (3,) respectively were found in two strains.

Table IV
Characterization of plasmids of multiresistant P. mirabilis strains

No. of strains	Serogroup (No. of strains)	Plasmid pattern (Mdal)	Transconjugants (Mdal)
1	O30	1.8, 22, 62	—
2	O18, NT	1.8, 22, 62, 103	103 (Am, Cb, Azl, Mz)*
36	O11 (2), O18 (23), O26 (3), O27 (1), O30 (1), O42 (1), NT (5)	22, 62	—
5	O18	22, 62, 103	103 (Am, Cb, Azl, Mz)
5	O3 (1), O18 (1), O23 (2), O26 (1)	5.0, 22, 62	—
1	O18	5.0, 22, 62, 70	—
1	O18	5.6, 22, 62	—
2	O26, NT	5.0, 38	—
1	NT	5.0, 38, 42, 70	—
2	O18	38, 70	—
1	O18	70, 113	113 (Am, Cb, Azl, Mz, Cm, Gm, Tm, Km, Nm)
1	O18	17.5, 32, 70	—
1	NT	17.5, 42, 52, 80	17.5 (Am, Cb, Azl, Mz, Cm, Km, Nm)

* For abbreviations see Table I
 NT = nontypable

Plasmid screening. Plasmid patterns and the results of transfer experiments are presented in Table IV. Two plasmids with 22 and 62 Mdal molecular weight were found in 85% of the strains, and 60% of the isolates contained only these two ones. Neither these most frequently occurring plasmids nor some other plasmids proved transferable in conjugation experiments without mobilization. Only 3 plasmids determining resistance to several antibiotics could be transferred into *E. coli* recipient as shown in Table IV. The sensitive strains harboured no plasmids.

Discussion

Bacteria of the tribe *Proteeae* are a common cause of hospital-acquired infections, particularly those of the urinary tract [14]. Among them *P. mirabilis* is the most frequent nosocomial pathogen [13, 15–18]. Previous reports of multiple-drug resistance of *Proteus*, however, have been largely concerned with *Proteus* spp. other than *P. mirabilis* [1, 19–23]. Infections or nosocomial outbreaks involving MR *P. mirabilis* have been infrequently reported [1, 24–26]. These cases were usually catheter-related urinary tract infections, and subsequent bacteraemias or infections of other body sites were also diagnosed. Data of Chow et al. [25] suggest that intestinal colonization may have been an important reservoir for MR *P. mirabilis* strains, and this finding may explain the difficulty encountered in the eradication and control of infections caused by them.

Although epidemiological analysis of cases was not involved in this study, it can be stated that the great majority (80%) of our 60 MR *P. mirabilis* were isolated from urine samples as well. According to the yearly report of Hungarian bacteriology laboratories, in miscellaneous clinical specimens urinary *P. mirabilis* isolates occur with the same frequency. The current increase in incidence of MR *P. mirabilis* has presented difficulties mainly in the treatment of urinary tract infections and consecutive infections. As drugs usually employed, including gentamicin and tobramycin, are ineffective against MR *P. mirabilis* (Table I), except for cefamandole and cefoperazone, second and third generation cephalosporins, amikacin, netilmicin and ofloxacin (and possibly other quinolones which were not examined) can be recommended as drugs of choice (Table II).

Interestingly, in the widely separated institutions, the same serogroup (O18) was found to be predominant among the MR *P. mirabilis* isolates (Table III). Strains belonging to this serogroup were not isolated at all in an early study in Hungary [13]. Moreover, in serogroup-distribution the present collection of strains differed sharply from isolates examined in 1956 (Table III).

All strains contained 2–4 plasmids in contrast to sensitive strains harbouring no plasmids. Presence of two plasmids (22 and 62 Mdal) in 85% of the isolates seems to be a characteristic feature of the MR *P. mirabilis* isolates. These plasmids proved nontransferable by plate mating experiments using *E. coli* recipient. Shafi and Datta [24] also found plasmids in MR *P. mirabilis* strains which were not transferable to *E. coli* K12 but could be transferred to another strain of *P. mirabilis*. Dance et al. [1] described an outbreak of urinary tract infection affecting 90 patients, that was associated with *P. mirabilis* resistant to gentamicin and several other drugs. The outbreak strain contained extrachromosomal DNA, but attempts to transfer this by conjugation have been unsuccessful. Verbrugh et al. [27] have found a plasmid encoding for multiresistance in several species of *Enterobacteriaceae* in a Dutch hospital. Gentamicin, beta-lactam and co-trimoxazole resistance could readily be transferred to *E. coli* recipient by filter mating. From the transconjugants a 60 Mdal plasmid was isolated.

Considering literary data, the plasmids widely spread in our MR *P. mirabilis* strains are likely to be responsible for multiresistance. Further investigations — including the use of another recipients — are needed in order to ascertain the precise function of these plasmids.

In spite of the findings that the great majority of the strains belonged to the same serogroup, produced the same antibiotic-inactivating enzymes, and possessed similar antibiotic resistance and plasmid pattern, there was no correlation between these markers. It means that a complex typing system is essential for differentiating strains of *P. mirabilis*.

Regarding the increasing importance of MR *P. mirabilis* infections in Hungary, there is a need for epidemiological investigations using an efficient typing method, control measures and an adequate antibiotic policy.

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NOTE ADDED IN PROOF

Ten representative strains were retested for production of aminoglycoside-modifying enzymes by Dr. Milan Kettner (Slovak Academy of Sciences, Bratislava, Czechoslovakia) using a radioenzymatic method. The results differ sharply from ours obtained by the method of van de Klundert et al. [6]. Dr. Kettner found enzymes only in 6 out of the 10 strains, 4 strains produced different kinds of acetylating enzyme together with a phosphorylating enzyme, and each of the remaining 2 strains had only one of these enzymes.

EFFECT OF HERBICIDES ON GROWTH OF SOME MICROSCOPIC SOIL FUNGUS SPECIES

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The effect of 14 herbicides and two herbicide combinations of three microscopic soil fungus species was investigated by Miller's method. (i) None of the 14 herbicides and 2 combinations belonging to four derivative groups induced the growth of *Aspergillus niger*, *Fusarium oxysporum* and *Rhizopus nigricans*; (ii) Higher doses of the herbicides prevented the growth of all test organisms significantly, the triasin derivative Hungazin PK being the less and tio-carbamate derivatives Alirox B 80 EC, Anelda Plus 80 EC and Anelda III being the most inhibitory. (iii) The sensitivity of test organisms against the examined herbicides decreased in the order of *Fusarium*, *Rhizopus*, *Aspergillus*.

A great number of papers have dealt with the biological effects of herbicides entering the soil. Many papers [1–4] call attention to the problem that herbicides kill not only the parasitical weeds, pathogens, noxious animals, but can damage also useful microorganisms living in the soil and taking part in transforming and decomposing processes. According to Müller [5], chemicals placed into the soil (insecticides, fungicides, herbicides) can be classified on the basis of their soil microbiological dynamics as stimulators, inhibitors and indifferent agents. A smaller group of herbicides stimulate microbiological activity in the soil [6–8], most of them, however, exert an inhibitory effect for a shorter or longer time [4, 9, 10]. Only few chemicals belong to the third group.

Microbiological activity in the soil can be measured by the quantity of CO₂ produced, by the enzyme activity of microorganisms, by the quantitative and qualitative composition of microflora or their changes. Baklivanov, Lalova [6] Lyszenko, Ivtinskaya [11], Anderson et al. [12] and others have observed that the intensity of CO₂ production decreases during herbicide utilization. Schinner et al. [13] reported that enzyme activity strongly varied on the effect of herbicides (dinoseb, paraquat, 2, 4-D, 2, 4, 5-T, simazin and chloraxuron). It has been shown that not only the number of useful microorganisms decreases or increases in the soil as an effect of herbicides but the specific composition of microflora also changes [3, 13–18]. The influence of pesticides on the action of soil microorganisms can be studied under laboratory conditions, too. This paper gives an account of the results.

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Table I
List of herbicides

Name of herbicide	Agent	Doses applied ($\mu\text{g/ml}$)					
		1	2	3	4	5	6
I. Nitro derivatives							
1. H-108 A 33 EC	ethalfluralin	2.0	10.0	20.0	40.0	80.0	160.0
2. H-108 (combined herbicide)	+ Maloran 50 WP	3.0	15.0	30.0	60.0	120.0	240.0
	+ atrazin						
II. Carbamide derivative							
3. Maloran 50 WP	chlorbromuron	2.0	10.0	20.0	40.0	80.0	160.0
III. Triasin derivative							
4. Hungazin PK	actinit PK	1.2	6.0	12.0	24.0	48.0	96.0
IV. Tiocarbamate derivatives							
5. Vernolat 0 80 EC ant AD 67	vernolate	3.5	17.5	35.0	70.0	140.0	280.0
6. Vernolat I. 80 EC ant TI 35		3.5	17.5	35.0	70.0	140.0	280.0
7. Vernolat III. 85 EC ant. Növöki B	vernolate	3.5	17.5	35.0	70.0	140.0	280.0
8. Alirox A 80 EC ant AD 67 ant. EPIC		3.5	17.5	35.0	70.0	140.0	280.0
9. Alirox I. 80 EC ant. TI 35	vernolate	3.5	17.5	35.0	70.0	140.0	280.0
10. Alirox III. 85. EC ant. Növöki B	vernolate	3.5	17.5	35.0	70.0	140.0	280.0
11. Alirox 80 EC ant. AD 67	vernolate	4.0	20.0	40.0	80.0	160.0	320.0
12. Alirox B 80 EC	vernolate	4.0	20.0	50.0	80.0	160.0	320.0
13. Anelda Plus 80 EC	butylate	5.0	25.0	50.0	100.0	200.0	400.0
14. Anelda II. ant TI 35	butylate	5.0	25.0	50.0	100.0	200.0	400.0
V. Herbicide combinations							
15. H-108 A 33 EC+ Maloran 50 WP*		4.0	20.0	40.0	80.0	160.0	320.0
16. Alirox A 80 EC+ Vernolat A 80 EC*		5.0	25.0	50.0	100.0	200.0	400.0

* Equal parts

Materials and methods

Herbicides. The 14 herbicides and two herbicide combinations we used in the course of our investigations are shown in Table I.

Organisms. The three microscopic fungi (*Aspergillus niger*, *Fusarium oxysporum*, *Rhizopus nigricans*) were isolated from calcareous chernozem soil type.

The effect of herbicides on the growth of fungus colonies was studied by Miller's method [19]. Herbicides suspended in water at six different concentrations were incorporated into peptone-glucose agar (Bengal-rosa) and discs inoculated with the above-mentioned fungi were put onto the plates. In this way the effect of herbicides was estimated by measuring the diameter of the fungus colonies after three to six days incubation and the results were evaluated by analysis of variance.

Results

The results of eight parallel examinations for each of the 14 herbicides and two herbicide combinations showed that none of the 14 herbicides and two herbicide combinations stimulated the growth of the test-organisms. The inhibitory effects observed were not significant in any case according to the analysis of variance.

Table II shows that the treatments, as compared to the control, showed an average inhibiting effect of 84.7%. *F. oxysporum* proved to be the most sensitive, being inhibited even by the dose used in practice with the exception of

Table II
Frequency of inhibiting effect of herbicides on test-organisms

Herbicides	Test-organisms (<i>Aspergillus</i> , <i>Fusarium</i> <i>Rhizopus</i> , number of significant inhibitions)			Number of inhibition, sum total
1. H-108-A 33 EC	3	6	5	14
2. H-108-C	5*	6	4	15
3. Maloran 50 WP	6*	6*	2	14
4. Hungazin PK	4	6	2	12
5. Vernolat A 80 EC	3*	6	6	15
6. Vernolat I, 80 EC	3	5*	6	14
7. Vernolat III, 85 EC	5*	5	6	16
8. Alirox A 80 EC	4	6	6	16
9. Alirox I, 80 EC	4	6	6	16
10. Alirox III, 85 EC	3	6	6	15
11. Alirox 80 EC	3	6	4	13
12. Alirox B 80 EC	5	6	6	17
13. Anelda Plus 80 EC	6	5	6	17
14. Anelda III.	5	6	6	17
15. H-108 A + Maloran	6	6*	5*	17
16. Alirox A + Vernolat A	6	4	6	16
Sum total	82 (85,4%)	91 (94,8%)	71 (73,9%)	244 (84,7%)

The inhibiting effect for the most part (224) was significant at level $P = 0,1\%$, but in some instances* (8) it was significant only at level $P = 5\%$

four agents (Vernolat III, 85 EC, Anelda Plus 80 EC, Alirox + Vernolat). An inhibitory effect occurred in 91 cases out of the total 96 ones, i. e. in 94.8%. This proportion was 85.4% with *A. niger* and 73.9% with *R. nigricans* i. e. inhibition occurred in 82 and 71 cases out of 96, respectively.

Rhizopus was not affected by the carbamide derivative Maloran 50 WP and by the triasin derivate Hungazin PK. In the case of this organism a significant inhibitory effect was shown against the two herbicides only in the highest doses. The sensitivity of the test organisms decreased in the order of *Fusarium*, *Rhizopus*, *Aspergillus*. Hungazin PK belonging to the triasin derivatives showed the least inhibiting effect. This herbicide inhibited *F. oxysporum* in 100% (i. e. in all of the six concentrations), *A. niger*, however, only in the two highest doses (33.3%). Consequently, Hungazin PK had an inhibiting effect in 66.6% in all of the cases. Hungazin PK was followed by Alirox 80 EC (72.2%) and by H-108-A 33 EC, Maloran 50 WP, and Vernolat I. 80 EC (77.7%). The highest inhibition was caused by Alirox B 80 EC, Anelda Plus 80 EC, Anelda III. and H-108-A 33 EC + Maloran 50 WP (94.4%).

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